THE CHEMISTRY AND BIOCHEMISTRY OF LYSINE RESIDUES IN PROTEINS

A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in Chemistry

at the

University of Canterbury

by

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2004

Itaque Claudius iaci pullos sacros in mare iussit, dicens, "Fortasse bibent quod edere nolunt!"

Adapted from Suetonius, Life of Tiberius

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Abstract

This thesis investigated the nutritional consequences of processing chicken feed, in particular the loss of available amino acids as a result of the Maillard reaction, both in model protein systems and during poultry feed processing.

Model systems containing RNase A and pure or feed-type carbohydrates were incubated at 37°C, 50°C or 70°C for up to 8 days. From lysine analysis, reduction in reactive amino groups in RNase A was shown to occur rapidly at room temperature, with no incubation period necessary. However, protein fragmentation that occurred during incubation, especially at higher temperatures, interfered with the quantification of lysine. SDS-PAGE showed crosslinking reactions of RNase A with the tested carbohydrates to be slow below 70°C. Incubation of RNase A with cyclotene or xylose produced the greatest rate of crosslinking, while starch and sucrose produced the least.

A modified OPA method was developed such that the level of reactive lysine could be quantitated in barley flour proteins, in a manner that was technically straightforward, inexpensive and allowed good through-put of samples. This method was shown to have good agreement with the published ninhydrin method in the measurement of Maillard reacted lysine in barley flours. Up to 25% loss of reactive lysine was observed in barley flour that had not undergone processing beyond milling.

Samples were taken before, during and after the pelleting of chicken feed. Lysine analysis of these samples showed loss in amino group content of at least 18% could occur during processing, although this was dependent on variations between pelleting runs. Protein fragmentation during processing potentially masked further losses.

A growth trial assessed a novel method of lysine addition to the feed, via spraying on free lysine solution post-pelleting. Over the 2 week trial period, 10% of the lysine applied by this method remained in the uneaten fines. Lysine eaten by chickens aged day 8-14, as calculated from measurements taken using the OPA method, correlated well with bird growth. Increased feed intake was also seen as the lysine content of the diet increased. In chickens aged day 15-21, lysine addition above the first addition level produced no further significant gain in performance. Therefore, lysine was not growth limiting above this level. Results indicated that over the 2 week period of the trial, equivalent performance for birds on the standard feed could have been achieved with a 50% reduction in free lysine added to the feed formulation. No lysine loss occurred in the standard sample as a result of pelleting.

Therefore, while the potential exists for lysine loss to occur during chicken feed pelleting, this thesis has shown that this is not a significant problem. As up to 25% lysine blockage in unprocessed barley flour was observed, obtaining feed ingredients with consistently low levels of Maillard reaction damage may be as important as maintaining ideal processing conditions.

Abbreviations

°C	degrees Celsius
adj	adjusted
amino gp/kg	amino groups per kilogram
AMPS	ammonium persulfate
Arg	arginine
Cf	frozen control
Ci	incubated control
cm	centimetre
Da	Daltons
DBC	dye-binding capacity
dDBC	differential dye-binding capacity
dH_2O	distilled water
DHA	dehydroascorbic acid
DM	dry matter
DMSO	dimethyl sulfoxide
DNP	dinitrophenyl
DTT	dithiolthreitol
F1	finisher 1
FCR	feed conversion ratio
FDNB	1-fluoro-2,4-dinitrobenzene
FDNBd	FDNB difference
g	gram
g	gravity
g/L	grams per litre
kDa	kiloDalton
kg	kilogram
kPa	kilopascals
kW	kilowatt
L	litres
Lys	lysine

M	molar
mA	milliamp
mg	milligram
mg/mL	milligrams per millilitre
MIU	o-methylisourea
mL	millilitre
mm	millimetre
mM	millimolar
mOPA	modified OPA
MWCO	molecular weight cut off
$NaBH_4$	sodium borohydride
nm	nanometre
OPA	o-phthaldialdehyde
ppm	parts per million
RNase A	ribonuclease A
s	seconds
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethylthylenediamine
TGase	transglutaminase
TNBS	trinitrobenzene sulphonic acid
Tris base	tris(hydroxymethyl)aminomethane
USA	United States of America
UV	ultra violet
v/v	unit volume per unit volume
W	watts
w/v	weight unit per volume unit
w/w	weight unit per weight unit
Wh/kg	Watt hours per kilogram
µg	microgram
µL	microlitre

Acknowledgements

Dr Juliet Gerrard – thank you for your tremendous supervision, support and endless enthusiasm. You have collected a remarkable selection of people into your group, and it has been fabulous to be a part of it.

Thank you to Agmardt for the funding which allowed me to do this research, it was much appreciated. In particular, thank you to Susan Christie from Agmardt for sorting out airfares, conferences and anything else that was required. Thank you to Canterbury University for providing additional funding.

Huge thanks to Tegel Foods Limited, for joining the project with enthusiasm, samples and growth trials. Thank you to Graeme Coles for your support, advice and help with the chicken side of the project. Thanks also to the rest of the Crop and Food team for flour samples, sample testing and advice.

Thank you to all of the exceptional people that have been part of, or associated with, Juliet's biochemistry team over time. In particular:

Sian Fayle, for paving the way and helping get me started.

Jackie Healy, for all of your freely imparted knowledge on all manner of techniques, and patience with my piles of chicken feed.

Renwick Dobson, for your support and always keeping me entertained.

The lovely Paula Brown, for the inspiration of those perfect gels and that perfect hair.

Susie Meade, for always being superb for advice and good for a gossip. Your help and support with the chickens, pH stat and proofing has been much appreciated.

Antonia Miller, for your proofing, assistance and enthusiasm. You are a gem!

Matt Walters, for keeping me company in the early days, for taking great photos of gels and for making me famous on all sorts of departmental advertising material.

Thanks also to the fabulous bunch of friends and flatmates who have kept me sane and entertained.

Thank you to my family, for all your support, help and belief. I couldn't have done it without you.

And finally, thank you to George for your encouragement and support over the years. It has been much appreciated.

INTRODUCTION

CHAPTER 1

1.1 OVERVIEW

This thesis examines the effect of animal feed processing on the nutritional value of proteins, with a particular focus on the effect of the reaction between reducing sugars and amino groups on this process. In animal feed processing, any loss in feed quality may have detrimental effects on animal productivity, and economic returns.

1.2 THE IMPORTANCE OF PROTEINS

Every living organism requires proteins for virtually every facet of life. This importance of proteins has long been recognised, with the word "protein" originating from the Greek word "proteios", which means "of the first rank or importance".[1] Today, the term encompasses an enormous group of organic polymers, small to large, simple to complex, which are vital to the majority of biological processes. Every cell, whether plant or animal, depends upon proteins to perform a large and diverse range of tasks – including enzymatic catalysis, the transport and storage of ions and small molecules, muscle contraction, structural support, storage mechanisms of vital nutrients, the generation and transmission of nerve impulses and protective mechanisms for the organism.[2]

The polymeric nature of proteins allows this group of organic molecules to have an almost infinite range of structures, and hence to carry out this prolific variety of roles within living organisms. The monomeric unit of the protein is the amino acid. In general, an amino acid consists of a central tetrahedral carbon atom covalently bonded to a carboxylic acid group, a primary amino group, a hydrogen atom and a variable side chain (Figure 1.2-a).

It is the orders of these side chains in each peptide chain, and the precise manner in which the peptide is folded, which gives each protein its unique properties.[2,3]

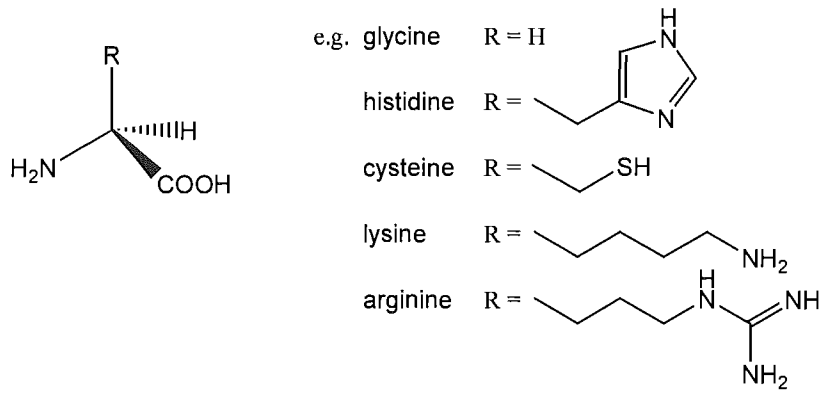

Figure 1.2-a: The general structure of the L-α-amino acid, with the side chains of some nutritionally important amino acids.

1.3 PROTEINS AND NUTRITION

Due to their ubiquitous nature, proteins are a common and vital component of many foods, where, as well as being a nutritional necessity, they affect colour, flavour, texture, and palatability.[4] Plants are able to synthesise all of the amino acids required for making the proteins required for their life cycle. In contrast, animals are only able to synthesise certain amino acids, and must include the remaining amino acids in their diet.[5] These amino acids, which cannot be synthesised by animals, are known as nutritionally essential amino acids (Table 1.3-a).[5] The particular amino acids which are essential also differ between animal species.[5,6]

Animals cannot store excess dietary amino acids for more than a few hours, and all proteins in the body are continually being synthesised and degraded.[7] Therefore, the essential amino acids must be incorporated into the animal's diet, through the digestion of other animal or plant protein sources, on a regular basis.[5] If an animal's diet is inadequate, such that it does not contain sufficient amounts of any one of the essential amino acids, the growth rate of the animal reduces or stops completely, and protein from

body tissues is reallocated to vital areas.[7] Hence, the ratio of essential amino acids, within a protein source, is the major determinant of protein quality.[5]

	Essential			Non-essential		
Human	Histidine Isoleucine Leucine	Lysine Methionine Phenylalanine	Threonine Tryptophan Valine	Alanine Arginine* Asparagine Aspartic Acid	Cysteine* Glutamic Acid Glutamine* Glycine*	Proline* Serine Tyrosine*
Chicken	Arginine Histidine Isoleucine Leucine	Lysine Methionine Phenylalanine	Threonine Tryptophan Valine	Alanine Asparagine Aspartic Acid Cysteine*	Glutamic acid Glutamine Glycine*	Proline* Serine* Tyrosine*

* Conditionally essential amino acids: a dietary source of the amino acid is required under conditions where endogenous synthesis cannot meet metabolic need.[6]

Table 1.3-a: The classification of the twenty most common amino acids according to the dietary requirements of humans[6] and chickens.[5]

In general, a high quality protein source can be defined as protein that contains adequate amounts of all the essential amino acids an organism requires, in a digestible form.[8] Protein digestibility is defined by the ability of the constituent amino acids to be absorbed through the gut wall of the animal.[5] Various enzymes are responsible for the digestion of protein into its constituent amino acids, but these enzymes cannot break up peptide bonds if they are not accessible. Hydrochloric acid in the gut serves to denature many proteins, allowing better access by enzymes. This process is still imperfect however, and amino acids from most food sources are not completely digested.[5]

In foods, many factors can affect protein quality along with the amino acid composition and digestibility (Table 1.3-b). These factors can be regarded as intrinsic to the protein, such as the source of the protein, the processing and storage history of the food, or the presence of antinutritional properties within the protein itself.[8] For example, several proteins contained in soybeans are known to inhibit growth, interfere with trypsin digestion of proteins, cause enlargement of the pancreas, and interfere with the absorption of dietary fats in the young chick.[5] Other factors are extrinsic to the protein. These extrinsic factors

include variables such as the energy content of an animal's diet, other constituents contained within the diet, and bacterial or toxin contamination, along with factors such as the species of the consumer of the protein.[8]

Protein related	Amino acid composition, primary through to quaternary structure, stability, interactions, digestibility, inherent antinutritional qualities
Processing history	Alkali, chemical, heat or pressure treatment etc., storage, contamination by bacteria, presence of antinutritional factors
Dietary	Total protein, total calorific intake, dietary fibre, frequency of feeding
Consumer related, physiological	Species, age, gender, reproductive status (pregnancy and lactation), general health and pathological states (trauma, stress, neoplasia)
Sociological	Economic, hygiene, and sanitation

Table 1.3-b: Factors affecting protein quality. Adapted from Owusu-Apenten.[8]

1.3.1 Protein quality and human nutrition

In New Zealand, like most developed countries, the majority of the population obtains sufficient high quality protein, as our varied diet incorporates high quality protein foods such as meat, eggs and fish.[9] Cereals, however, are the major source of protein for the majority of the earth's population.[4] Like most foods of plant origin, cereals are of limited value as they are deficient in some essential amino acids, particularly lysine.[10] This can lead to a major problem in third world countries, where ensuring the population is receiving adequate protein can be far more difficult than meeting basic energy requirements. Rice, for example, is the major food eaten in parts of Asia, Africa and South America, but it only contains about 5% protein, and this protein is not easily digested.[4] Hence, even assuming all the protein was digested, the average 20 year old, 72 kg male would need to eat almost 3 kg of cooked white rice daily to meet the United States National Research Council's recommended dietary allowance for protein.[11]

It is not only in the poorer areas of the world that protein quality is an issue. Despite the majority of the population of developed countries receiving enough high quality protein,

certain sectors are at risk. Vegetarians, and especially vegans, must incorporate foods containing complementary proteins into their diet.[6] For example, wheat is most limiting in lysine, but contains adequate amounts of methionine.[11] Beans are most limiting in methionine, but contain adequate amounts of lysine, and hence are a complementary protein source to wheat.[11] Including both in the diet increases the protein quality of the diet, although not to a quality as high as a diet containing fish, milk or meat.[11] In addition, the complementary proteins need to be consumed at approximately the same time to be as effective as a high quality protein, as proteins cannot be stored by the body.[11]

Pregnant or breastfeeding women and people on restricted or low calorie diets should also be very aware of the quality and quantity of the protein they are consuming, as inadequate dietary intake of high quality protein can detrimentally affect health and growth.[8] This is also a major consideration for nutritionists formulating diets for certain sectors of society in developed countries, where diets are restricted, such as babies fed on infant formula or commercial baby food, some elderly and those on liquid or intravenous diets.[8] It is also recommended for athletes to increase high quality protein intake, and many amino acid and peptide supplements are produced in an attempt to meet these requirements.[1]

1.3.2 Protein quality and livestock nutrition

Diet formulation and protein quality is of particular importance to sectors involved with animal production, and the intensive livestock industry in particular. Feed is the major cost in animal production,[12] and millions of metric tonnes are manufactured every year to meet industries' need to produce eggs, poultry and pig meat, dairy, beef and fish.[13] In 1998 alone, 758.2 million tonnes of animal feed was used worldwide, and this is projected to rise to 1172.4 million tonnes in 2010.[13]

Much research has been undertaken to find the specific nutritional requirements of many animals. This research has resulted in many tables and books outlining the nutritional needs of many species of farmed animals.[14] More information is continually being published about the interactions between various ingredients in the diet, and between diet and factors such as animal age, sex, reproductive status, and housing conditions such as

temperature, humidity and airflow.[15-21] In chapter 4, the dietary requirements of poultry are discussed in greater detail.

Inadequate levels of essential amino acids within diets leads to diminished animal growth and performance.[5] However, research has shown that adequate levels of the essential amino acids do not ensure maximised growth, as some non-essential amino acids are synthesised from essential ones. For example, in chicken metabolism, tyrosine is classed as a semi-essential amino acid, as it is synthesised from phenylalanine, which may be in limited supply. Similarly, cysteine is derived from methionine, and hydroxylysine from lysine.[5] In addition, interactions between different amino acids can exist.[5]

Interactions between amino acids can be classed as imbalances or antagonisms. Imbalances in the levels of essential amino acids can be detrimental to animal growth. For example, if a diet is changed so that the difference between the first and second limiting amino acids increases, the rate of animal growth decreases.[5] Antagonisms can occur between various amino acids with similar structural or chemical characteristics, such as leucine and isoleucine, arginine and lysine, and threonine and tryptophan.[5] This antagonism occurs due to the increased level of one of the amino acids having a detrimental effect on the metabolism of the other.[5] For example, if lysine is the first limiting amino acid, increasing the level of arginine exacerbates this lysine deficiency. This occurs because lysine and arginine share intestinal transport and renal reabsorption systems, and these systems have limited capacity.[7] Toxic effects may also occur if the level of one amino acid is excessively high compared to the other amino acids.[7] This toxic effect is unlikely to occur unless pure amino acids have been added to the feed mix.[7]

While there now exists a large body of literature on the nutritional needs of a variety of animals, research into this area is continuing. In the poultry industry, for example, the role of amino acids in the immune response is under investigation,[22] as is the potential for non-standard food ingredients to replace more traditional diets, for reasons of economic viability,[13] and the growing public distaste for meat and bone meals being fed to animals.[22] While many factors that influence the growth rate of animals are continuing to be found, protein quality and content in the diet will always be a major consideration.

1.4 THE REACTIVITY OF PROTEINS

It cannot be assumed that any diet, which has been constructed in raw form to meet all dietary protein needs, will remain unchanged until the time of consumption. However, this assumption is sometimes made.[23] As there are many possible reactions that can occur in food and feed systems during both processing and storage, it is vital that these effects are taken into account if an animal's growth is to be maximised through a nutritionally complete diet. Hence, it is of great importance to understand the range of chemical reactions open to proteins under *in vitro* conditions associated with processing and storage, and how these influence nutritional quality and animal performance.

1.4.1 The reactivity of proteins within organisms

In general, systems are in place *in vivo* to regulate the reactions of proteins.[24] This is necessary as the array of sidechains within the 20 common amino acids allows for a wide variety of possible chemical reactions. Most of the reactions that do occur are necessary for cell function. Cysteine, for example, contains a thiol group that can react with another thiol to form a disulfide bond, acting to stabilise the tertiary structure of proteins (Figure 1.4-a (i)). The side chain of lysine contains an aliphatic primary amino group, which can undergo reaction with aldehydes and ketones to form imines, also referred to as Schiff bases (Figure 1.4-a (ii)).[25]

Many proteins exist in a conjugated form, *i.e.* protein that has been modified post-translationally, to include covalently bonded non-amino acid groups. The most common form of post-translational modification of proteins in cells is thought to be glycosylation, the enzymatically controlled covalent bonding of carbohydrate groups to polypeptide chains to form glycoproteins.[28] This reaction is considered to affect at least half of all proteins in a eukaryotic cell,[28] and most commonly involves the hydroxyl group of serine, threonine or hydroxylysine, or the amide nitrogen of an asparagine residue.[3,29] Some proteins can also be covalently linked to lipid molecules or to nucleic acids.[30] Metal atoms, heme, phosphate and flavin can also be found bound to certain proteins.[2,3]

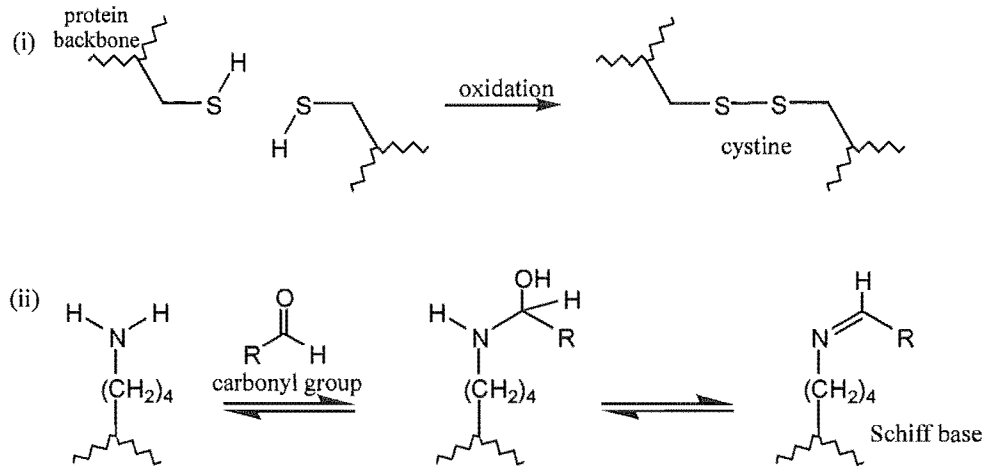

Figure 1.4-a: (i) Formation of the disulfide bond of cystine from two cysteine residues.[26] (ii) The reaction between a lysine residue and a carbonyl group to form a Schiff base. Adapted from Fayle and Gerrard.[27]

Reaction of proteins *in vivo* can also lead to disease states. These can occur when regulatory pathways controlling the protein folding breakdown,[31] or when long-lived protein undergo reaction over the lifetime of an organism.[32] For example, glycation, the non-enzymatic covalent attachment of carbohydrates to protein, has been linked to diseases such as diabetes mellitus, non-diabetic nephropathy, macrovascular disease, Alzheimer's disease, cataract formation and ageing.[32]

1.4.2 The reactivity of proteins under processing and storage conditions

When proteins are removed from their native environment, as happens when plants are harvested and processed for food purposes, the proteins are subjected to a wide range of conditions that would not be encountered *in vivo*. These *in vitro* conditions can include physical treatments such as milling or heat, biological treatments such as fermentation or enzymatic hydrolysis, and chemical treatments such as the use of an oxidising agent or an alkali.[33,34] This can often result in the denaturing of the protein, so that the protein is

unfolded from its normal conformation to form a random coil with many possible conformations, exposing reactive groups.[4]

Furthermore, the concentrations of many metabolites changes when metabolism is shut down. New compounds may be introduced and a wide range of chemistry becomes available to the protein that was inaccessible *in vivo*. Fats and their oxidation products, polyphenols, vitamins B_6 and C, alkali and reducing sugars can all react with protein bound amino acids, to give products that may enhance flavour and colour, be toxic, or cause loss of nutritional value.[33] This loss of nutritional value is generally associated with reactions with the essential amino acids lysine, methionine, cysteine and tryptophan.[33] It has been stated that, apart from the destruction of particular vitamins, the reactions that proteins undergo are the major chemical reactions that occur during food processing.[35]

Under alkaline conditions, or when heated, crosslinking of protein can occur, along with amino acid racemisation and the destruction of some amino acids.[33] Protein crosslink formation impacts on nutritional quality, as further discussed in section 1.5.3, and can be due to the reaction of lysine residues with dehydroalanine, the product of a β-elimination from cysteine, serine, threonine or phosphoserine, to give lysinoalanine.[4,36] Severe heating may lead to isopeptide bond formation, due to the formation of peptide bonds between the ε-amino group of lysine and the amide groups in asparagine or glutamine residues.[36]

Unsaturated lipids can undergo oxidation in the presence of heat, light or catalysts, to form hydroperoxides, aldehydes, ketones, and carboxylic and polymerisation products.[36] The first three of these can react with proteins and modify some amino acids. Hydroperoxidases can oxidise the essential amino acids methionine, cysteine and tryptophan residues, whereas the aldehydes and ketones are most reactive with the amino group of lysine residues.[33] Quinones can also react with methionine, cysteine, tryptophan and lysine.[33] Quinones are produced from polyphenols that have been oxidised under alkaline conditions, or in the presence of the enzyme polyphenol oxidase.[4]

Of all the possible reactions that proteins may undergo during food processing and storage, the reactions between reducing sugars and proteins are thought to be the most prevalent,[36] and are the major cause of the degradation of the nutritional quality of protein in food.[36] These so called Maillard reactions are discussed in detail below.

1.5 THE MAILLARD REACTION

The term "Maillard reaction" encompasses a complex network of reactions, initiated by the reaction of an amino group with a carbonyl group, that results in the formation of a plethora of products that are vital to the flavour, aroma and appearance of foods.[37] The reaction was first noted in 1912 by Louis-Camille Maillard, who observed, on heating a mixture of amino acids and sugars, that a yellow/brown colour was formed.[38] Due to this colour that develops during later stages of the Maillard reaction, this group of reactions is often referred to as non-enzymatic browning by food chemists. This is in contrast to enzymatic browning, such as occurs in cut bananas, pears and lettuce as a result of polypolyphenol oxidase action on polyphenol compounds in these foods.[39] In medicine, the Maillard reaction is often termed glycation, or non-enzymatic glycosylation.[37,40-42]

The initial stage of the Maillard reaction, where an amino group, such as that in lysine, reacts with the carbonyl group, often from a reducing carbohydrate, results in the formation of a Schiff base (Figure 1.4-a(ii)).[43] In some instances, the Schiff base produced undergoes subsequent rearrangement to form a product reported by M. Amadori in 1931,[44] now known as the Amadori product. Figure 1.5-a shows these initial reactions in more detail.

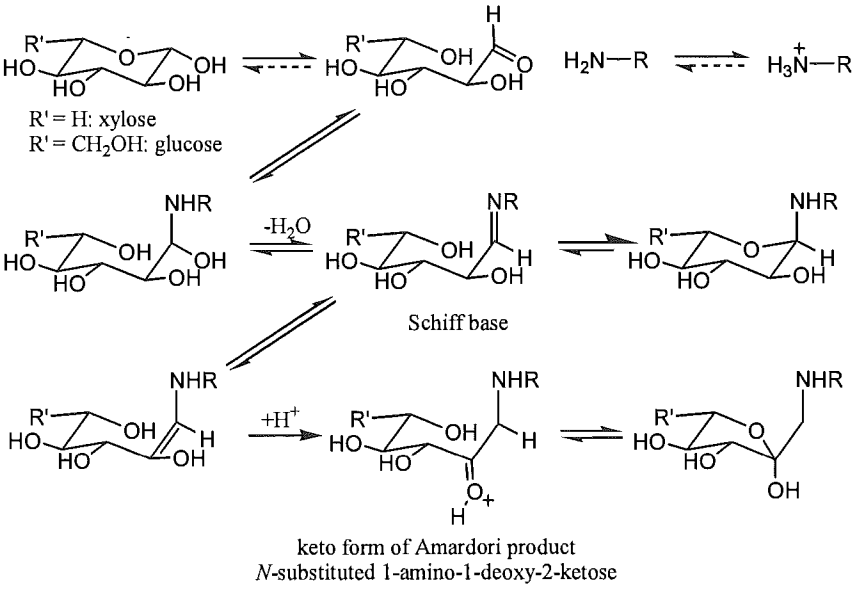

Figure 1.5-a: The initial reactions of the Maillard reaction to form the Amadori product. Adapted from Bristow.[45]

Subsequent to the formation of the Amadori product, a wide range of reactions can occur, dependent on conditions such as pH, water content, temperature and other molecules present.[46] These reactions encompass the intermediate stages of the Maillard reaction, and can include rearrangements, cyclisations, dehydrations, retroaldolisations, fissions, isomerisations, and further condensations.[47] Ultimately, in the advanced stages of the Maillard reaction, melanoidins are formed – high molecular weight, brown nitrogenous polymers and co-polymers.[46] These are also known as advanced glycation end-products, or AGEs, particularly in the medical field.[46]

These reactions were summarised in 1953, when John Hodge published a scheme of the Maillard reaction (Figure 1.5-b).[48] This basic understanding of the Maillard reaction is still widely utilised today.[47] In addition, there have recently been reaction pathways discovered, leading to advanced Maillard reaction products, which do not occur *via* the Amadori product.[47]

There are many difficulties inherent in the study of the Maillard reaction, due to both the complex nature of the reaction and the vast variety of potential products, which can vary from those of a low molecular weight, to large, macromolecular and crosslinked structures.[46] Hence, while research into the Maillard reaction has been undertaken for many years, it is only more recently that the structures of some Maillard reaction products have been elucidated.[46] To further complicate the investigation of this group of reactions in human food and animal feed, a few micrograms of a molecule may be enough to give a food product its distinctive aroma or flavour, or to inhibit a gastric enzyme.[43] Consequently, low yield Maillard reaction products can be more significant than the more easily identified major reaction products.[46] Even the major reaction products can be very difficult to isolate, as isolation methods may alter the product, or advance the reaction along a different path.[47 49]

As L.-C. Maillard astutely noted ninety years ago, sugars and amino acids are prolific in nature, and hence any reaction between them would have far reaching consequences.[38] Today, the Maillard reaction is extensively studied in diverse range of fields, predominantly in food and medical science.[37,46]

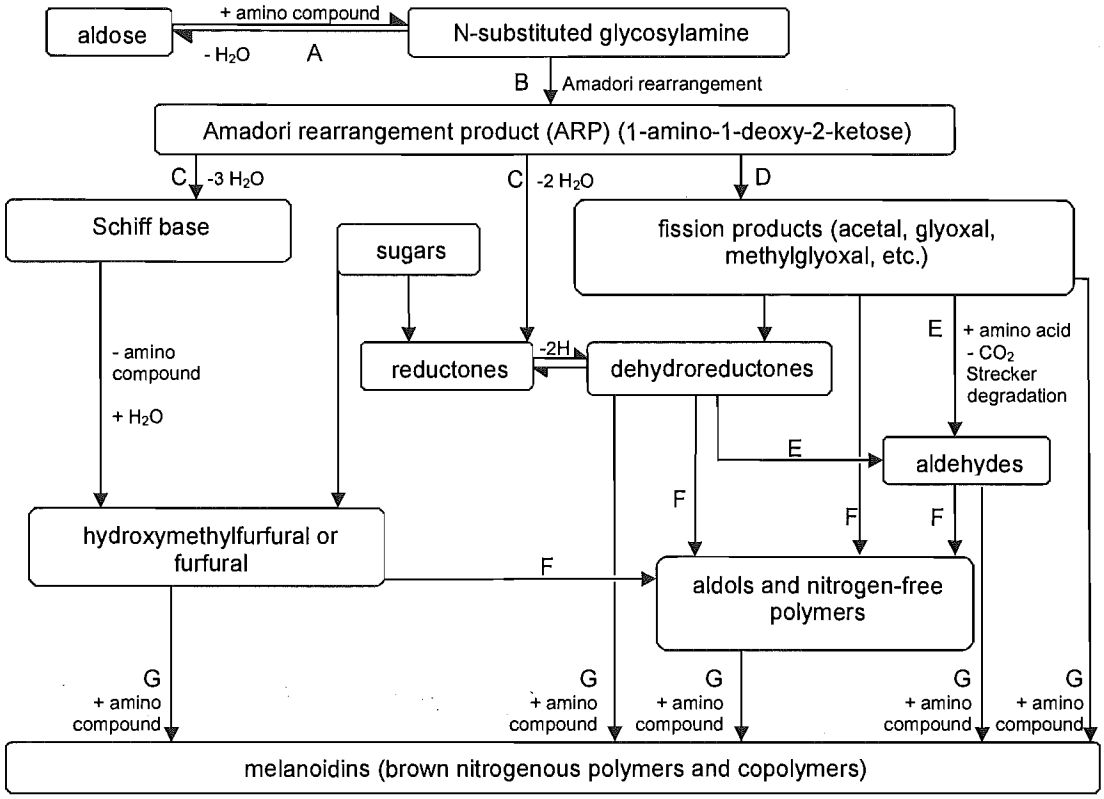

Figure 1.5-b: The Hodge scheme of the Maillard reaction. Adapted from Ames,[47] based on the original Hodge scheme.[48] A – sugar-amine condensation; B – Amadori rearrangement; C – dehydration of Amadori product; D – fragmentation of sugar moiety; E – Strecker degradation of amino acid moiety; F – aldol condensation; G – aldehyde-amine polymerisation and formation of heterocyclic nitrogen compounds.[48]

The Maillard reaction in medicine

The occurrence of the Maillard reaction within the human body was first discovered during the study of human haemoglobin, when a modified form of haemoglobin, containing a covalently bonded low molecular weight adduct, was found and later identified.[43,50] This glycated form of haemoglobin was discovered to exist in higher levels in the blood of diabetics, leading to further research in this field.[43]

In diabetics, the normal production of the hormone insulin is retarded, necessitating the control of blood glucose levels by diet or by injected insulin.[3] Neither of these methods is able to mimic the natural feedback response adequately, and hence the level of blood glucose is often higher than in non-diabetics.[3] This accelerates the rate of the Maillard reaction, which is now thought to account for many diabetic complications.[51] Maillard reaction products have also been associated with non-diabetic nephropathy, macrovascular disease, Alzheimer's disease, atherosclerosis, cataract formation and aging.[32,52,53]

1.5.2 The Maillard reaction in simple systems

Many of the Maillard reaction studies undertaken have been in simple model systems, using an amino acid or dipeptide with a reducing sugar to determine the details of the Maillard reaction.[46] Fewer studies have been performed on whole protein systems, due to the increased complexity and the subsequent difficulties in the study of these systems.

Studies undertaken using simple model systems have given a basic understanding of the initial stages of the Maillard reaction, and insights into the intermediate and late stages.[46] Many products have been isolated from the Maillard reaction of simple model systems, some of which are often used to indicate the progression of the reaction in more complex food systems. Hydroxymethylfurfural, furosine, N^{ε}-carboxymethyllysine and lysinoalanine are the most common indicators used in these systems, with the first three indicating early, intermediate, and advanced Maillard reaction occurrence respectively, and lysinoalanine indicating protein crosslink formation (Figure 1.5-c).[46,54] A variety of other products isolated after Maillard reaction in simple model systems are also shown in Figure 1.5-c.

After advanced Maillard reactions have occurred in model systems containing simple sugars and amines, products are formed with molecular weights of 7 kDa and greater, due to the polymerisation and crosslinking of low molecular weight compounds.[43] As a consequence of the large number of very similar products obtained after Maillard reaction of even simple systems, it is very difficult to isolate these high molecular weight products, and only recently has progress been made in this area.[46]

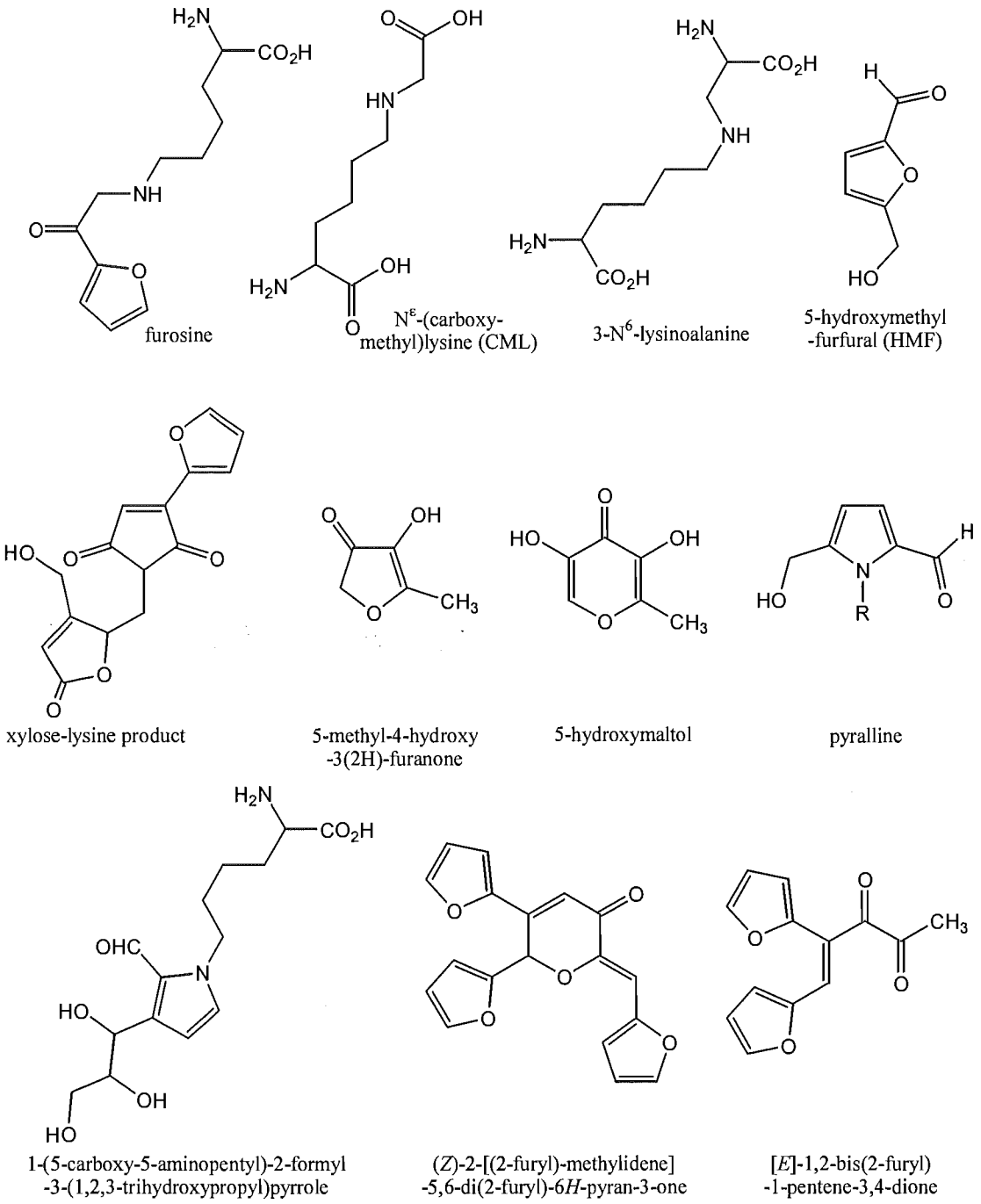

Figure 1.5-c: Some of the more common compounds used to indicate the occurrence of the Maillard reaction,[54,57] along with a selection of other low molecular weight products isolated after the Maillard reaction has occurred, from in vitro *and* in vivo *systems.*[43,45,53,58-61]

Model systems have also helped elucidate the importance of lipid breakdown products in the Maillard reaction. In systems containing fat, both lipid oxidation and the Maillard reaction can occur, and each process can influence the other.[55] Carbonyl compounds produced during lipid peroxidation can react with amine groups in similar ways to the carbonyls present in sugars, producing macromolecules with melanoidin-like characteristics.[56] The addition of lipids to simple lysine/sugar systems has been shown to increase the rate of the Maillard reaction,[55,56] whereas Maillard reaction products have been shown to have an antioxidant effect, slowing lipid oxidation.[56]

There is now a general understanding of common pathways that the Maillard reaction often takes, for example *via* reactive α-dicarbonyl moieties, such as glyoxal and methylglyoxal.[62,63] Both of these intermediates have been shown to be formed during the early Maillard reaction, for example glyoxal can be formed directly from the autooxidation of glucose and unsaturated fatty acids,[63] but the exact mechanisms of formation are still open to conjecture.[62,63]

α-Dicarbonyls

Recent research has shown the vital role that α-dicarbonyl compounds play in protein modification in both the food and medical fields (Figure 1.5-d).[64] These highly reactive α-dicarbonyl species can be produced by the autooxidation, cleavage, isomerisation, cyclisation or dehydration of sugars.[63,65] While caramelisation of sugars alone does produce α-dicarbonyls, the Maillard reaction has been shown to increase the levels of α-dicarbonyls formed.[66]

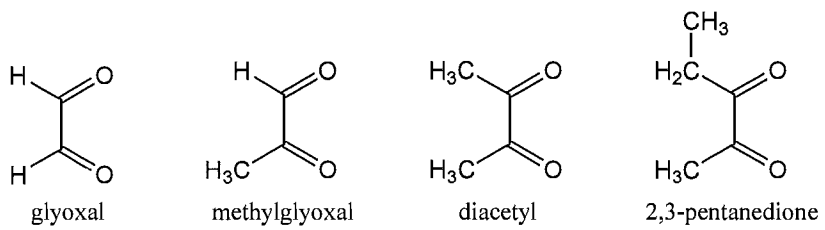

Figure 1.5-d: A selection of α-dicarbonyl compounds formed during the Maillard reaction.[65]

Recent work has been undertaken to determine precisely how these α-dicarbonyls are formed during reaction, both in terms of the pathway of production[65-68] and the factors affecting the concentrations produced.[66,69] ^{13}C labelling has been an invaluable tool in determining the origins of these α-dicarbonyl species.[65,67,68,70,71] For example, in Figure 1.5-e, four different formation pathways of methylglyoxal from a glucose Amadori product are shown.

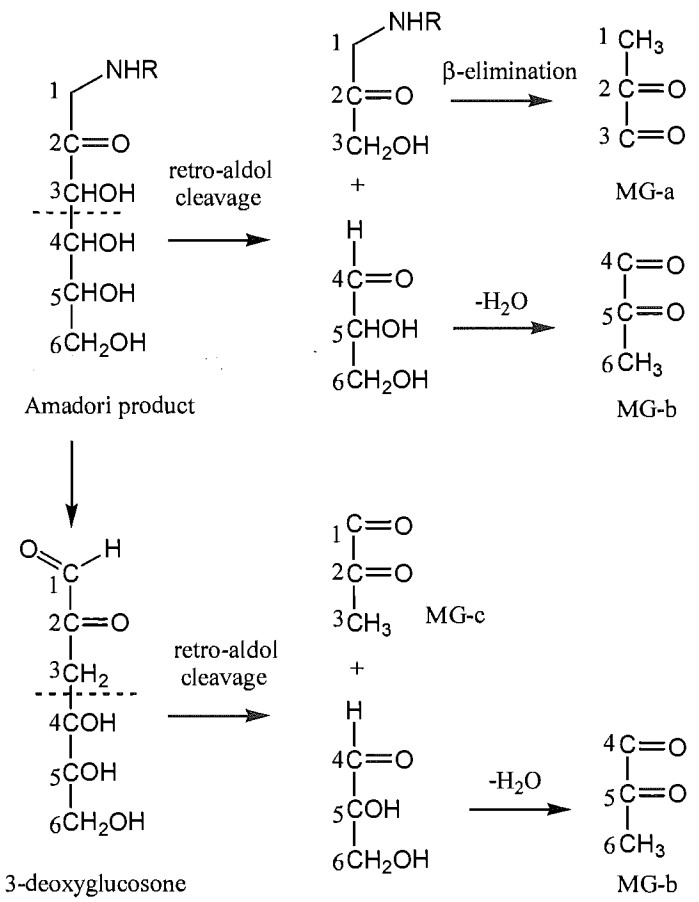

Figure 1.5-e: Alternative routes for formation of methylglyoxal (MG) from an Amadori product formed from glucose, with carbon numbers indicating original D-glucose carbon positions, from ^{13}C studies – MG-a, b and c refer to the different possible methylglyoxal molecules formed based on these original D-glucose positions. From Yaylayan and Keyhani.[65]

Methylglyoxal has been shown to have extremely high reactivity with proteins, leading to the formation of crosslinked products almost instantaneously.[72] While compounds such as diacetyl and glyoxal have very similar structures to methylglyoxal, the rate at which they crosslink proteins is much lower.[72] Research is being carried out to try and account for the different reactivity rates seen with dicarbonyl compounds.[73]

1.5.3 The Maillard reaction in proteins

While simple model systems have greatly broadened our understanding of the Maillard reaction,[43] it may be inaccurate to relate these results to the reactions that occur in whole proteins, under *in vivo* or food processing conditions.[46] In food science, the study of the Maillard reaction in proteins has mainly been limited to the effect on nutritional and functional properties of the food. Therefore, while there is much literature concerning the effect of heat on the nutritional value, texture, flavour, aroma, and colour of food, there is little that examines the specific chemistry occurring within the food proteins that give rise to these effects.[49] However, both medical and food researchers have recently focussed on the effect of the Maillard reaction on proteins *in vivo*, which has resulted in some key insights into the chemistry occurring in a variety of systems.[49,74,75]

In proteins, the primary amino of lysine residues is the most reactive group under most circumstances.[43] The N-terminus amino group can also react, and has been shown to react to form the Schiff base more rapidly than lysine residues, although lysine residues react to form the Amadori product more quickly.[76] The side chains of residues other than lysine have also been shown to partake in the Maillard reaction. Arginine, with its guanidine group, is often reported in the literature for its involvement in Maillard reactions, both *in vivo*[77-80] and *in vitro*.[81-84] There are a number of papers detailing the formation of crosslinks within proteins involving both lysine and arginine residues.[77,83,85] However, arginine does not appear to react significantly with reducing sugars such as hexose, requiring more reactive moieties, such as dicarbonyl compounds, to undergo Maillard reaction.[43] Proline, containing a secondary amino group, is a major amino acid in cereals, and may also undergo reaction.[43] The levels of protein-bound tryptophan, histidine,

tyrosine, cysteine and methionine have also shown to be reduced after incubation with glucose.[86]

Crosslinking of proteins

The crosslinking of proteins is very important in the formation of HMW products.[87] Even with small proteins, the formation of dimers, trimers, tetramers and greater can quickly lead to large insoluble structures.[88] Some of the crosslinkages that have been found in model systems are shown in Figure 1.5-f.

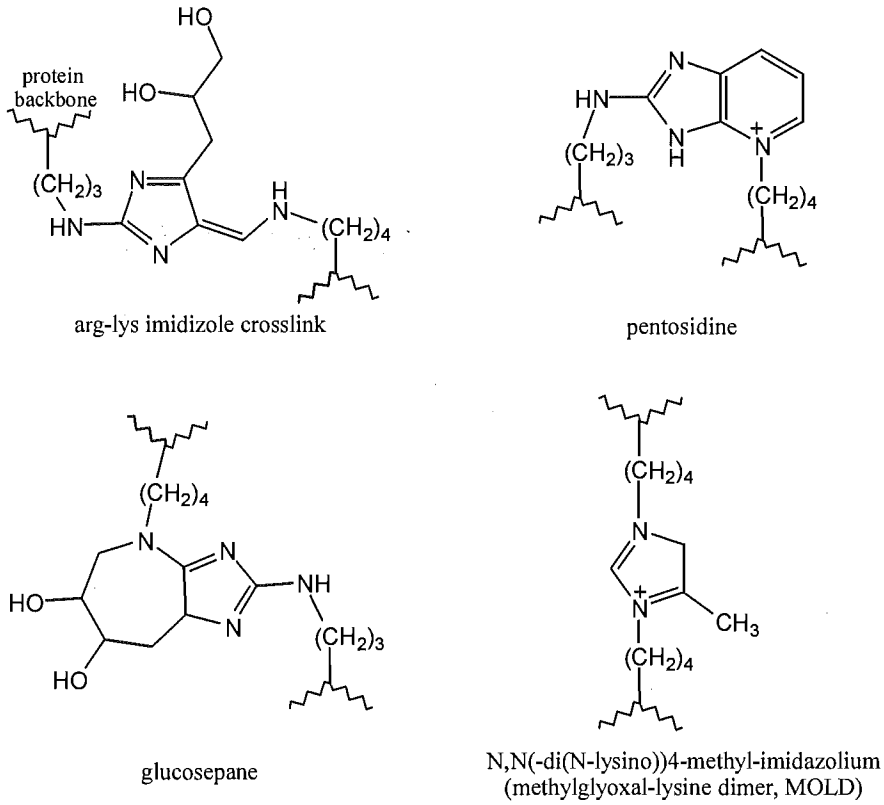

Figure 1.5-f: A selection of crosslinkages isolated after Maillard reaction in model systems.[85,87,104-106]

The crosslinkages that occur between proteins is of great interest to many researchers, whether the aim is to prevent or reverse them in medical applications, or to utilise them for the formation of novel foods.[89-93] In medicine, the crosslinking of proteins is closely

associated with the pathology of the disease states associated with the Maillard reaction. For example, in cataract formation the formation of crosslinks within the lens protein has been associated with the resulting opaque nature of the eye lens.[94,95] Similarly, treating collagen under Maillard type reaction conditions has led to increased stiffness in the collagen network.[96] It is proposed that such an increased stiffness *in vivo* could contribute in osteoarthritis and diabetic-dependent cardiac disease.[96-98] Crosslinked proteins have also been associated with amyloid plaques in the brains of Alzheimer sufferers, and may be associated with other neurodegenerative diseases.[99,100] Increasingly, age and diabetes related disorders are being associated with elevated levels of Maillard reaction products, such as carboxymethyllysine and pentosidine.[46,101-103]

While the study of protein crosslinking *in vivo* is a relatively recent endeavour, the effects of crosslinking on food proteins have been studied for many years. A good review of the various types of crosslinking which can occur in food systems is given in Gerrard.[107] These include disulfide crosslinks, and crosslinks derived from dehydroprotein, tyrosine, and the Maillard reaction.[107] The impact of these various types of crosslinks on food properties can be profound and include effects on the texture and nutritional quality.[107]

1.5.4 The importance of the Maillard reaction in food and feed

Consequences of the Maillard reaction for food acceptability

Properties of food such as texture, colour, aroma and flavour are of great importance to the food manufacturer and the home food preparer, as these factors determine how acceptable the food is to the consumer. Much research has been devoted to the determination of how flavour and aroma are altered in food, as a result of the Maillard reaction.[37]

The low molecular weight products of the Maillard reaction are often associated with the flavour and aroma of cooked or processed food. For example, pyrazines appear to be directly associated with the nutty and roasted flavour and aromas of many foods, cyclic enolones confer caramel aromas, while burnt aromas can arise from polycarbonyls.[108] In some model systems, low molecular weight products have been shown to be almost solely

responsible for colour formation.[109] Hofmann has shown that most coloured products of glucose/amino acid mixtures, heated under typical cooking conditions, have molecular weights of less than 1000 Da.[110]

Much of the research into the affect of the Maillard reaction on the sensory properties of food products has focused on the volatile components formed during reaction, and it is only more recently that the properties of non-volatile sensory active compounds have been studied.[111] These studies have identified compounds that enhance sweetness, without being sweet themselves;[111] compounds that have a cooling effect, without the typically associated mint flavour;[112] a compound that has a umami-like (savoury) flavour,[113] and compounds that are detectably bitter with concentrations as low as 0.25 μM in water.[114]

A goal of much of the research into the identification of Maillard reaction products with various sensory characteristics is to increase acceptability of foods by consumers.[43,115-119] This can be achieved by controlling the formation of these compounds by altering processing conditions.[47] In conjunction, protein crosslinks are important in relation to the formation of flavour, aroma and texture in foods, and hence of great interest to food manufacturers.[107] The ability to form new products, and to enhance existing products by altering crosslink patterns in food proteins is an area of great research potential.[107] Some of the research occurring in this area includes investigating how the Maillard reaction can be used to make gels or films from dried egg white,[120] cottonseed protein,[121] bovine serum albumin,[122] and whey protein.[91] The effects of protein crosslinking, *via* the Maillard reaction, have also been studied in relation to the functional properties of fish myosin protein,[123] and wheat doughs.[124]

In the poultry industry, the importance of taste on feed acceptance has been the basis of a number of studies.[125] Chickens are known to be very good at adjusting dietary intake to maintain a stable energy intake over varying diet compositions,[126] however they do not have a highly developed sense of taste or smell, possessing only 24 taste buds.[125] While chemical agents unpalatable to chickens have been discovered, no flavouring agent has been found which increases feed intake of a typical well-balanced poultry feed.[125] Therefore, potential exists for Maillard reaction products to be unpalatable to chickens, given that low concentrations can be bitter to humans,[114] no research appears to have

focussed on this area. However, the potential for the Maillard reaction to reduce the nutritional quality of chicken feed has received mention in some texts.[127-130]

Nutritional consequences of the Maillard reaction

The loss of nutritional quality during processing and storage has been well documented in a wide variety of foods, from infant formulas and milk products to royal jelly.[39,131-134] This decrease in nutritional quality is due to the modification of individual amino acids, predominantly lysine, and a potential decrease in the overall digestibility of protein. This can be due to the formation of protein aggregates during the Maillard reaction, as the digestive enzymes may fail to recognise their substrate or cannot reach the point of action due to solubility or denaturation limitations, and because of potential inhibition of proteolytic and glycolytic digestive enzymes by products of the Maillard reaction.[36]

Additional loss of nutritional quality may occur due to the formation of antinutritional and toxic compounds during the Maillard reaction.[39] Some products of the Maillard reaction that have been shown to have mutagenicity are shown in Figure 1.5-g. However, Maillard reaction products have also been shown to be beneficial, having antioxidant,[135-137] anti-mutagenic,[138,139] and antimicrobial properties.[140-142]

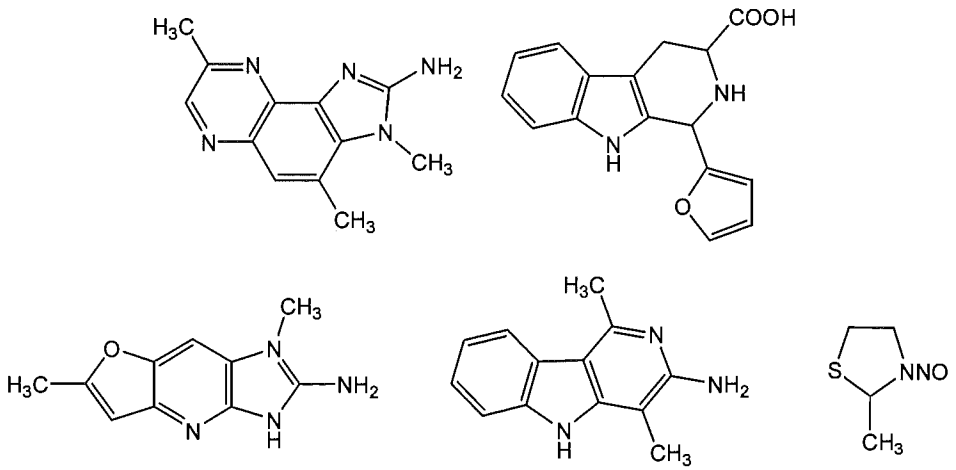

Figure 1.5-g: Maillard reaction products shown to be mutagenic.[43,108,143]

The degree of damage to individual amino acids and whole proteins during the Maillard reaction can be manipulated by altering processing conditions. Minimising processing and storage temperatures and times, altering starting pH and ingredient composition, are amongst possible methods to reduce the effect of the Maillard reaction on the nutritional quality of food and feed.[47,133,144,145] Similarly, the concentrations of anti-nutritional or beneficial products formed during the Maillard reaction can also be changed by altering these factors.[39,146]

An essential aspect of determining the affect of the Maillard reaction on food or feed, and hence understanding the effect of altering processing conditions, is the measurement of the degree of nutritional damage that occurs during this processing.[35] Many reviews investigating the nutritional damage in Maillard reacted foods extensively cover the impact of the loss of lysine availability and the measurement of this loss.[10,35,108,147] This is because lysine is the amino acid most affected by the Maillard reaction, and also the most difficult to assess for nutritional availability.[35] In chapter 2, the difficulties with testing for lysine are covered, and the methods available for testing lysine contents are reviewed.

The relevance of the Maillard reaction to the intensive livestock industry

Any effects of the Maillard reaction on protein quality or the amino acid profile of feed is highly relevant to the intensive livestock industry. Within New Zealand, the poultry industry is large, and growing to meet consumer demand. In 2000, poultry meat accounted for 30.6% of all meat consumed in New Zealand, on average 28 kilograms per person – an increase of 7.6% compared with 1999.[148] The New Zealand chicken industry employs around 3000 people, and earns nearly $500,000 in retail sales.[148]

Worldwide per capita poultry consumption is also increasing, with a 1.5% annual increase estimated until 2009.[22] The United States of America, China, Brazil and the European Union are the largest consumers and the greatest producers of poultry worldwide, accounting for 75% of global production.[22] Within these groups, annual per capita growth is estimated to be between 1.1% for Brazil and 5.1% for China.[22]

In such a competitive environment, everything must be as streamlined as possible, to maximise profit. The major cost in poultry production is the raw material for feed.[149] In the

USA in 1999, feed ingredient costs alone represented 52% of live production costs.[150] As noted in section 1.3, a high quality protein source is particularly important for maximising animal growth. Broiler (meat chicken) diets are very dependent on imported protein sources, primarily soybean meal, to meet these protein requirements.[151] Synthetic amino acids are also routinely added to poultry diets, with lysine the most common supplement.[5]

Poultry diets are pelleted to improve animal performance[12] in a procedure involving pressure, moisture and temperature, all of which could contribute to Maillard reaction, as further detailed in chapter 4.[152] The continual requirement of companies in the intensive livestock industry to increase profit has led to studies specifically investigating the nutritional value of heat-treated feed ingredients and processed diets for animals.[153-155] These are discussed in chapter 4. Overall, the potential for Maillard reaction to occur during feed processing is well accepted.[156]

1.6 OVERVIEW OF THESIS

Interest in the Maillard reaction is increasing in the medical field, as recognition of its importance in the pathology of many diseases is realised. However, the food industry has acknowledged its importance in flavour and colour formation in processed food for decades. The loss of nutritionally available lysine during the Maillard reaction, and the damage to proteins, has also been accepted for many years.[157,158] The importance of the Maillard reaction is well established, and research aimed at understanding its reaction pathways and the implications of products formed is continuing in many fields.

For the intensive livestock industry, any factor resulting in diminished economic returns must be minimised. The potential of the Maillard reaction to occur under feed processing conditions is high. What is not fully understood is the degree to which this occurs, and, if it is a problem, how to minimise it. Methods for testing the progress of the Maillard reaction in foods can be expensive, time consuming and misleading. Methods of better defining the progress of the reaction in foods, and identifying the products, are continually being refined. However, much work remains to be done.

This thesis is concerned with both gaining a better understanding of the progress of the Maillard reaction in model protein systems, and determining its relevance for the poultry industry in New Zealand. As the limiting amino acid in poultry diets is lysine, the primary focus of this research is the reaction of lysine under these conditions. Specifically, this research will focus on whether the Maillard reaction occurs under feed processing conditions, and how this can be controlled, either through changing processing conditions or by adding lysine post-processing.

Chapter 2 will review the methods for lysine testing in the literature, and will focus on development of a method to monitor lysine content in model systems, food and feed. Methods for testing for arginine will also be discussed.

Chapter 3 will look at model studies of the Maillard reaction under controlled conditions, with simple protein and sugar systems, with the aim of developing a better understanding of lysine and arginine loss in relation to protein crosslinking.

Chapter 4 will focus on model systems based on barley flour, sugars and lysine, to determine what potential exists for Maillard reaction to occur under feed pelleting conditions.

Chapter 5 will examine the results from a feed trial, looking at alternative methods of lysine addition to feed, carried out at the Monogastric Unit at Massey University, and the implications for feed processing.

Chapter 6 will summarise the thesis, and chapter 7 details experimental processes.

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MONITORING THE MAILLARD REACTION – METHOD DEVELOPMENT

CHAPTER 2

2.1 BACKGROUND

Chapter 1 has established the importance of lysine to the intensive livestock industry, and the potential for the destruction of lysine to occur during processing and storage as a result of the Maillard reaction. Hence, a method of accurately analysing the degree of lysine loss that occurs during the processing of animal feed as a result of the Maillard reaction was required.

There are many methodologies available for assessing various aspects of the Maillard reaction and its products.[1] One common method utilises the colour change that generally occurs in foods that have undergone Maillard reaction, which is also known as non-enzymatic browning. Therefore, the increase in absorbance at 420-480 nm can be used to give a coarse estimate of Maillard reaction progression. However, the degree of browning has been shown to be dependent on both sugar type and amino acids present,[2] and protein damage can occur prior to browning becoming visible.[3] This thesis is primarily concerned with the nutritional consequences of the Maillard reaction cascade, and hence methods that are nutritionally based are most relevant. As lysine is both an essential amino acid and, as discussed in chapter 1, the amino acid residue most susceptible to the Maillard reaction due to its primary amino group, the main focus of this research concerns its loss as a nutritionally available moiety. A variety of methods for analysing this lysine loss, along with related methods for following the progress of the Maillard reaction, including crosslinking rate, arginine content and pH change, are reviewed in this chapter. In addition, this chapter describes the adaptation of methods for examining the Maillard reaction in systems examined in chapters 3-5.

2.2 LYSINE TESTING METHODOLOGY – LITERATURE REVIEW

If the nutritional value of a human food or animal feed is to be determined, it is important to establish the concentrations and digestibilities of nutritionally relevant constituents. As discussed in chapter 1, high quality protein is an essential part of the diet, and the quality of protein is significantly determined by the levels of essential amino acids contained within it. Lysine is one of these essential amino acids, and is present in growth limiting levels in many foods of plant origin, including cereals.[4] It is therefore particularly important, especially if Maillard reaction may have occurred, to establish an accurate estimation of the lysine content in order to determine the nutritional value of the food or feed.

Quantifying lysine content is problematical, and much literature examines and expands on the methodology best suited to this task. For the purpose of this thesis, a lysine measuring method that was rapid, involved a relatively simple chemical test, and was relevant to chicken nutrition was required. Reviews of techniques for determining lysine contents of proteins include Freidman, 1982[4] and Owusu-Apenten, 2002.[5] Below, the various methodologies covered in the literature concerning lysine determination are summarised and compared.

The main priority with all amino acid testing, with a nutritional aim, is to selectively assay amino acids that are relevant to human or animal nutrition. As shown in Figure 2.2-a, the total lysine content of food or feed is comprised of both lysine that is available for utilisation by the animal for growth and metabolism, and unavailable lysine, which the animal cannot utilise and subsequently excretes.[6] This lysine may be unavailable because the protein containing it is not digestible by the animal concerned, or because it has been chemically altered so it is no longer metabolised.[7] An ideal nutritional test will only measure the bioavailable lysine.[7]

The quantification of the available lysine content of food can be performed *in vivo* through animal testing, or *in vitro*, using chemical methods.[8] In general, *in vivo* testing gives a more accurate measure of the lysine content of a food, but tends to be expensive and time consuming. *In vitro* testing is quicker and cheaper, but less accurate than the *in vivo* approaches.[7] However, if an *in vitro* method is validated, using an *in vivo* method, for use

in a specific system the accuracy can be similar to that of an *in vivo* approach. With the variety of methods available, it is important to choose the technique most suited to the food or feed system under investigation, and often standard methods need to be modified to be specific for the protein or proteins under investigation. Some systems that are often tested for loss of nutritionally available lysine include infants' foods and formula, milk and dairy products, and many animal feed systems, especially those of the intensive livestock industries including poultry, swine and fish.[9-35] The solubility of the proteins, the moisture content, and the interference by other food components must all be taken into account when an assay method is chosen.

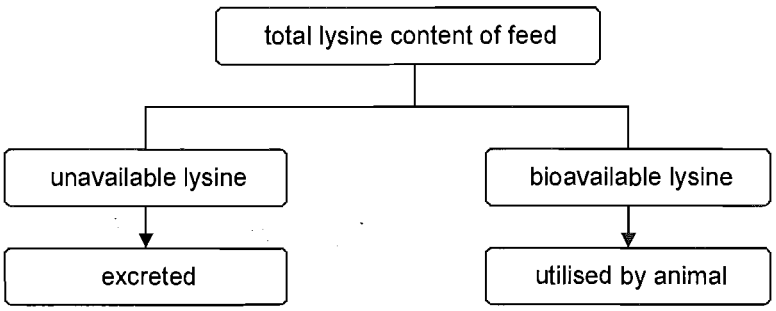


Figure 2.2-a: The difference between total, unavailable and bioavailable lysine. It is the challenge of lysine assays to accurately distinguish between these parameters.

Choosing an assay for the quantification of lysine in foods after Maillard reaction has occurred must be done with care. Since the mid 1940s, scientists have been aware that the reaction between reducing sugars and lysine results in products that revert back to free lysine after hydrolysis with a strong acid, but are not nutritionally available.[7] In particular, it is well established the Amadori product is not a biologically available source of lysine, but on reaction with acid it will hydrolyse to produce free lysine.[6] Hence, for an assay to be suitable for testing lysine in a situation where the Maillard reaction may have occurred, the assay cannot use an acid hydrolysis step to break down the protein prior to analysis, as commonly occurs in standard amino acid analysis.[8] Research has focused on developing lysine analysis methods that only measure the nutritionally available lysine, and not the nutritionally unavailable Maillard reaction products. In methods that utilise acid hydrolysis, nutritionally available lysine can be overestimated, sometimes very significantly.[8]

A method for determining lysine content must also be specific. In practice, this means the test must be able to reliably distinguish between lysine and other amino containing residues, especially arginine. Arginine contains a guanidinium group, which is fully protonated at neutral pH. The pK_a of this group is 12.5, as compared with the alkyl amino group of free lysine, which has a pK_a of 10.5.[36] These factors are utilised by some assays to differentiate between the residues. Many tests for lysine content also measure N-terminal amino groups, and this must be taken into account when analysing data. Significant protein breakdown can be an additional factor to take into consideration to avoid over-estimation of lysine levels.

The major aim of this thesis was to determine the significance of the Maillard reaction during the processing of chicken feed. In chapter 3, the loss of chemically reactive lysine is investigated in model systems. In chapter 4, the loss of nutritionally available lysine is assessed in model systems with conditions mimicking pelleting, and in large scale pelleting trials. Chapter 5 investigates whether this loss is significant for the chicken growth, through *in vivo* analysis. Therefore, techniques were required for analysis of lysine concentrations under each of these circumstances.

2.2.1 *In vivo* lysine determination

The optimal route to determining the efficacy of a food or feed as a nutritional source is, ultimately, to feed it to the animal for which it was designed. However, performance in animal trials may not always be related to the variable in question. What is generally required from these types of assays is a measure of the bioavailability of the variable under investigation. In the case of lysine, and other amino acids, bioavailability can be defined as "being in a form appropriate for digestion, absorption and utilisation."[37] Unfortunately, the determination of this bioavailability is rarely straightforward.[38]

One major complication in the determination of bioavailability through *in vivo* testing is sample variation.[8] Inter-animal genetic variation is a contributory factor to this, as is the difficulty in minimising variability in the diets of all factors other than those being investigated. Complications also arise from variables such as anti-nutritional factors in the diet, interactions between amino acids, feed intake, and the typically curvilinear response to the amino acid under investigation.[37] For example, if the Maillard reaction has occurred

to a greater degree in some feed relative to others, this may result in greater (or lesser) palatability, the formation of growth depressants and anti-nutritional compounds, or even the suppression of microbial activity.[39] Suppression of microbial activity in animals may have positive or negative consequences. If the microbes are utilised for the digestion of feed, such as in ruminants, this could negatively affect performance.[40-42] However, if the microbes are pathogenic, the suppression of their activity could have positive growth effects.[43]

A large number of methods have been developed for the determination of lysine availability by examining excreta, ileal contents or blood plasma.[37] With these assays, it is essential to differentiate between 'digestibility' and 'availability' as these terms are not interchangeable. Just because a moiety can be digested and absorbed, this does not mean it will be bioavailable under the definition given above, as it may not be in form which can be utilised. Most *in vivo* assays measure digestibility, not bioavailability.[37] It should also be noted that most *in vivo* assays require *in vitro* analysis of amino acid levels at some point in their methodology. In general, amino acid analysis is used in these assays to determine lysine levels, whether it is undigested lysine in the excreta, or lysine in the blood plasma.[37]

The digestive system of the chicken

When using *in vivo* assays for testing levels of amino acids for nutritional reasons, it is necessary to have a good understanding of the digestive system of the animal being studied, as this will affect the methodology that can be used and the significance of the result. For example, in cattle all enzymatic protein degradation that occurs in the rumen is of microbial origin.[44] Therefore, to gain a true understanding of the absorbable protein levels, duodenal cannulation is required, with separation of protein fed into ruminally degraded and undegraded protein.[45] As this thesis is concerned with the lysine requirements of the chicken, protein digestion in the avian digestive system, as shown in Figure 2.2-b, is discussed below.

In chickens, protein digestion begins in the proventriculus, with hydrochloric acid and pepsinogen being produced. Pepsinogen is converted to pepsin at low acidity, which results in the breakdown of proteins into peptides.[46] Mechanical grinding of the feed

occurs in the gizzard, allowing enzymes to access all portions of the feed.[46] Further hydrolysis of peptides occurs as the feed enters the small intestine. Peptides are absorbed into the mucosal *via* an active transport system, and this occurs mainly in the jejunum, whereas amino acids are mainly absorbed in the ileum.[46] Waste matter is excreted at the cloaca, which is common to the urogenital system also, and urine and faecal voiding occurs simultaneously.[37]

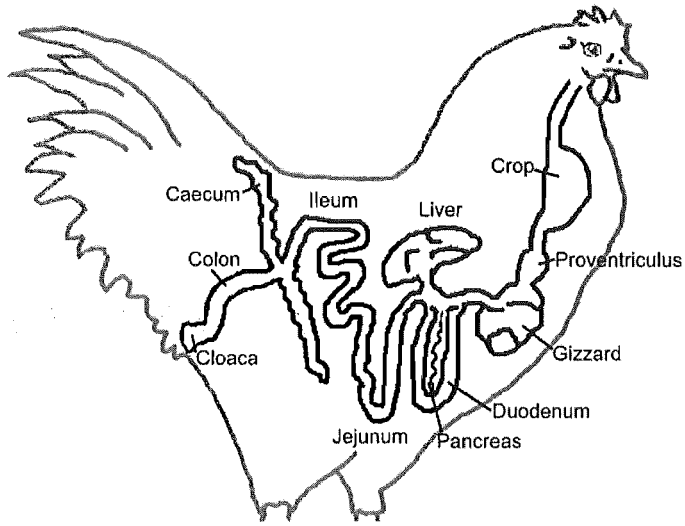


Figure 2.2-b: The avian digestive system. The small intestine contains the duodenal loop and the jejunum in the anterior portion, and the ileum in the posterior portion. Adapted from Leeson.[46]

2.2.2 *In vivo* techniques

Excreta digestibility

The technically simplest method, which can be used in growing or non-growing animals, is the excreta digestibility assay. In this assay, amino acid digestibility is assayed by measuring the amino acid levels fed to the animal, then subtracting the levels measured in

the excreta.[37] This method is straightforward to execute, and does not require the surgical modification or the death of the animals under study.[37,47] This methodology is made more complex in poultry, as faecal matter tends to be contaminated by urine, feathers and scales.[37] Most problematic, however, is the action of microbes in the hindgut, which mainly reside in the cacae.[37] These microbes may proteolyse and utilise amino acids not otherwise absorbed by the bird, resulting in misleading measures taken from animal excreta, and hence for the assay overall.[48,49]

Ileal digestibility

In an attempt to bypass the effects of hindgut bacterial action, ileal digestibility assays were developed.[49] Instead of measuring excreted amino acid levels, the contents of the ileum are collected and assayed. Two methods can be employed to collect ileal contents – killing the bird or cannula insertion (Figure 2.2-c).[37] Unfortunately, cannula insertion may interfere with normal physiological processes in the bird, leading to underestimation of most amino acid contents as compared with bird euthanasia.[37] In addition, cannulation of animals is expensive and labour-intensive.[50] Comparison between excreta and ileal measurements, in poultry, have shown considerable disparity, indicating hindgut bacteria do make a significant difference to excreted amino acid levels.[48]

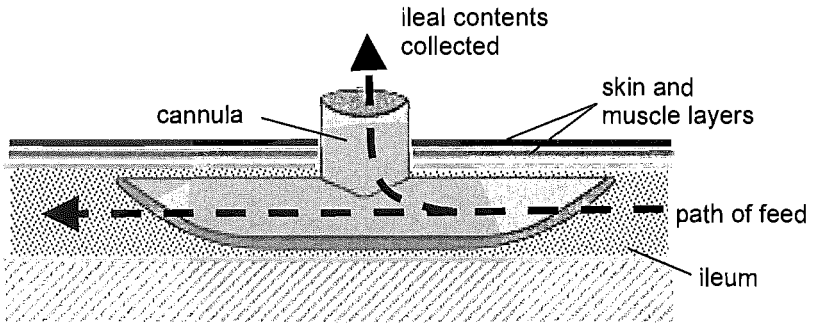


Figure 2.2-c: Schematic of cannula setup in the ileum. The cannula opening lies outside the body, and can be closed off when contents are not being collected to allow food to be digested normally. For testing, a bag can be attached to the opening, and a portion of the partially digested feed will collect within it. Adapted from van Barneveld.[51]

Traditionally, amino acid analysis has been used to assay the ileal content, but in 1996 Moughan and Rutherfurd published a technique using the MIU method (described below in Section 2.2.3) to determine the levels of reactive lysine in the digesta. This new method was developed due to concerns that the traditional true ileal digestibility study did not accurately estimate the availability of lysine in heat-processed feedstuffs.[38] Therefore, the Moughan and Rutherfurd method has been extensively studied for its suitability with Maillard reacted proteins.[23,38,52,53]

Plasma amino acid assay

Another method available to assess amino acids *in vivo* is the plasma amino acid assay, where the plasma levels of all amino acids are investigated on the assumption that the levels of free amino acids in the blood will reflect their dietary availability.[54] Unfortunately, plasma amino acid levels are dependent upon the nutritional status of the animal and are influenced, to a far greater degree than alternative methods, by such factors as amino acid imbalances, circadian rhythm, age, species, physiological status, meal size, feeding frequency, and ambient temperature.[37] For example, if the animal is in a positive nitrogen balance, anabolic processes remove the amino acids from the blood, but if the energy requirements of the animal are higher than nutritional supplies, catabolic processes will release amino acids into the blood.[37] It has been shown, however, that plasma free amino acid levels can be utilised to determine the limiting amino acids in poultry diets, and the method is considered relatively quick and convenient.[54]

Growth bioassays

According to Carpenter, growth bioassays are the only direct techniques available to determine the accuracy of nutritional values given by other procedures.[7] This is because growth is a response that incorporates the variety of components that can affect bioavailability – the digestion, absorption and utilisation of the amino acid. In general, the principle of these assays is to measure the ability of protein to replace a specific amino acid in supporting growth.[7] When the determination of the growth response to levels of an amino acid in which the diet is deficient is desired, graded levels of the amino acid are added to the deficient basal diet.[55] Often, other performance indicators are used in these

assays, in conjunction with growth rate, such as feed efficiency, amino acid intake, and empty (with intestinal tract removed) carcass gains.[7,56] However, despite the utility of this type of assay, growth assays are expensive and time consuming.[37] In addition, while suitable for assessing diets to be fed to growing animals, such as broiler chickens, growth assays are obviously less able to ascertain the dietary needs of non-growing animals, such as poultry used for breeding or egg laying.[37]

2.2.3 *In vitro* techniques for lysine determination

Due to the time consuming and expensive nature of bioassays, if a large throughput of samples is required or if facilities for animal feed tests are unavailable, *in vitro* chemical tests are generally more suitable. In these, the assumption must be made that chemically reactive lysine is equivalent to nutritionally available (bioavailable) lysine.[4,8] This relationship is shown in Figure 2.2-d. Often, the results of these determinations are described as 'reactive lysine' contents to acknowledge this limitation.[8]

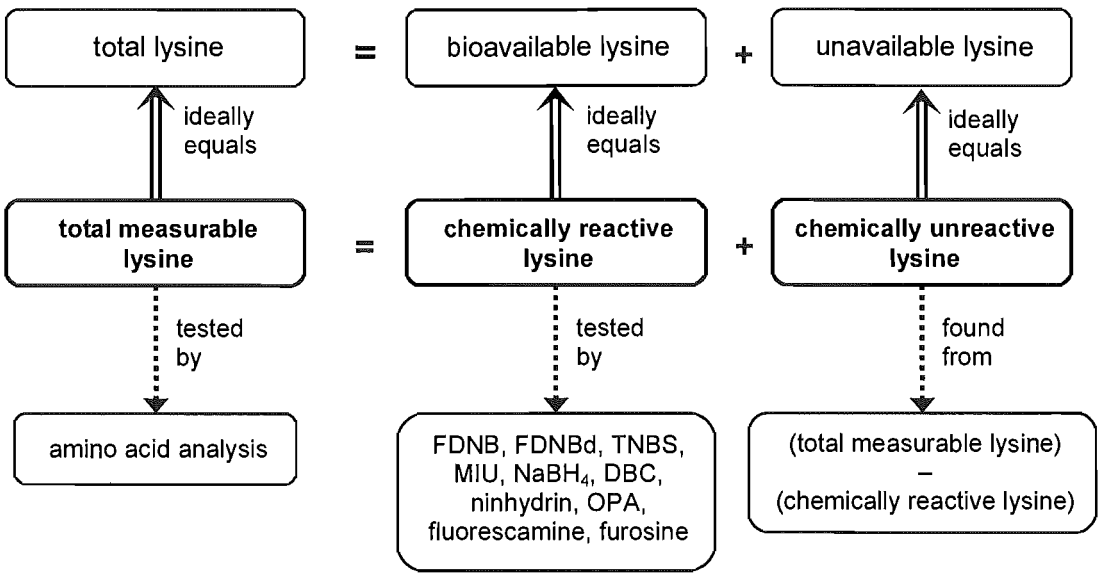


Figure 2.2-d: An overview of chemical analysis for reactive lysine. The major assumption made is that chemically reactive lysine is equivalent to bioavailable lysine. Abbreviations defined in text.

The primary approach to *in vitro* analysis of lysine content is through reaction of a chemical indicator with the ε-amino group of the lysine residue. The equivalence between reactive and bioavailable lysine will differ depending on the method used, the system undergoing testing, interfering constituents present, and the amount of Maillard reaction that has occurred in the system.[8] Sample preparation must also be taken into account. Poor sample preparation will affect the reliability of almost any method - a good review of the necessary steps of sample preparation is given by Finley.[57] Each sample, or group of samples, may have a method of lysine determination that is more appropriate, and this will also depend upon required throughput. The only way to find the true relevance of the test being used, in the particular system under investigation, is by animal trial. Unfortunately, this is rarely feasible.

Amino acid analysis

The common method used to determine the composition of protein in many foods is amino acid analysis. Amino acid analysis is an automated technique that involves breaking up the protein into constituent amino acids by using 6 M hydrochloric acid to hydrolyse all amide bonds.[8] Alternatively, a strong alkali can be used to achieve the same result.[57] Subsequent chromatography and spectrophotometric quantification of the resulting mixture determines the amount of each amino acid in the original protein. Due to the acid hydrolysis step, this method will generally overestimate nutritionally available lysine, but can be used to estimate 'total lysine' – *i.e.* both reacted and unreacted lysine content.[4] However, if the lysine residues have undergone more advanced Maillard reaction, free lysine may not be produced on acid hydrolysis, leading to underestimation of total lysine.[8]

FDNB method

In 1955, Carpenter and Ellinger developed the first method, now widely utilised, for measuring reactive lysine in food.[58,59] 1-Fluoro-2,4-dinitrobenzene (FDNB) reacts with the unreacted ε-amino groups of lysine to give dinitrophenyl (DNP) lysine after acid hydrolysis (Figure 2.2-e). This product is yellow and can be quantitated spectrophotometrically.[7] However, this method is time consuming and complicated, as FDNB is not water soluble.[8]

In addition, this method does not measure free or N-terminal lysine moieties, as these react to give di-DNP-lysine, which is removed during purification.[7] Coloured DNP products formed on reaction with arginine, hydroxylysine and ornithine present in the sample are not removed during this purification step, and chromatography is required if these are not to interfere with analysis.[7,8,60] Furthermore, if the sample contains high levels of polysaccharides, a 20-30% loss of DNP-lysine can occur during acid hydrolysis.[8] It appears this loss is due to the reductive, and hence decolourising, effect of sugar degradation products released during the hydrolysis step.[8]

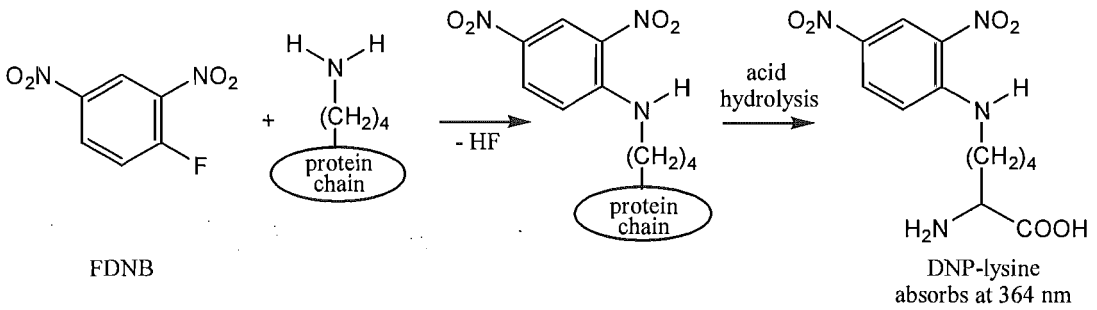


Figure 2.2-e: Reaction of FDNB with protein bound lysine. Adapted from Hurrell et al.[8]

FDNBd method

In an attempt to mitigate problems encountered with the FDNB method as a result of carbohydrate interference, the FDNB difference (FDNBd) method was developed.[8] This procedure assumes that the difference between total lysine, as measured by amino acid analysis, and reactive lysine, as measured by the FDNB method, would be the Maillard reacted (blocked) lysine that failed to react with FDNB (Figure 2.2-f). The blocked lysine, which does not react with FDNB to form DNP-lysine, is assumed to yield free lysine on acid hydrolysis in the same proportion as in the total lysine analysis. As DNP-lysine does not break down to free lysine, even if it is partially reduced during acid hydrolysis, free lysine can be measured and, on subtraction from total lysine, should give the amount of reactive lysine in the sample.[8]

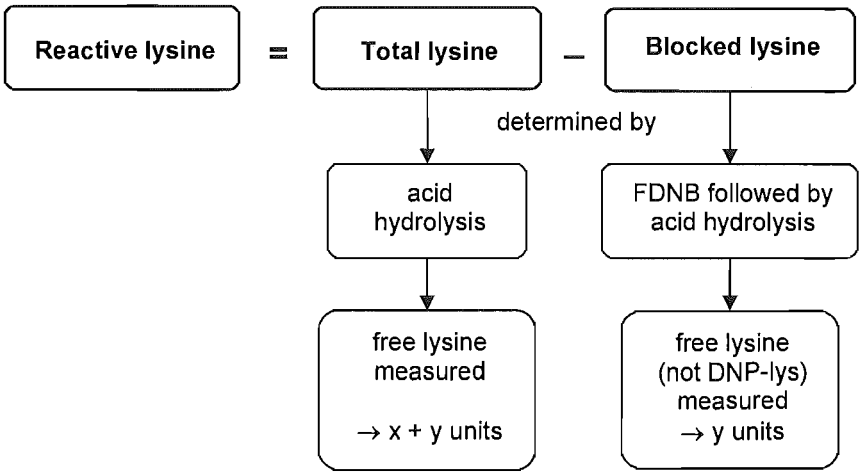


Figure 2.2-f: The determination of reactive lysine using the FDNBd method.

TNBS method

In an effort to improve upon the FDNB method, Kakede and Leiner developed the trinitrobenzene sulphonic acid (TNBS) method in 1969.[61] This reaction is very similar to that of the FDNB reaction – TNBS reacts with lysine to give trinitrophenyl (TNP) lysine (Figure 2.2-g), in a method examined in Cayot and Tainturier.[62] Unlike FDNB, TNBS has the advantage of being water-soluble, and in the TNBS method the acid hydrolysis step is shorter than in the FDNB method as only peptides, not amino acids, are required for reaction.[8] Furthermore, free arginine does not give a coloured product with this method. However, this method is more susceptible to interference from carbohydrates than the FDNB method.[8]

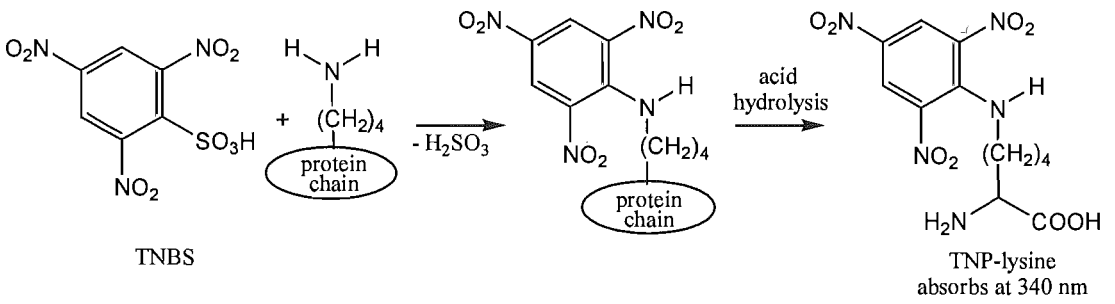
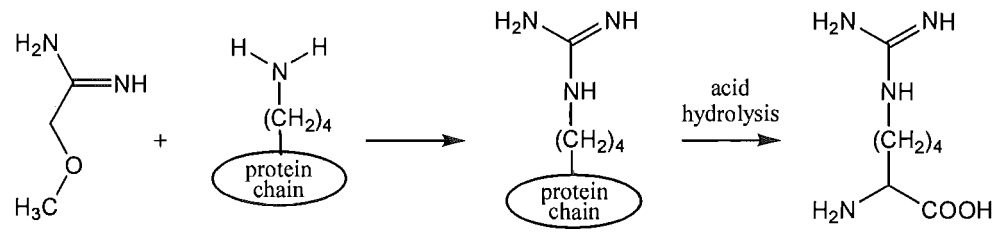


Figure 2.2-g: Reaction of TNBS with protein bound lysine. Adapted from Hurrell et al.[8]

MIU method

The *o*-methylisourea (MIU) method (also known as guanidation) was developed in 1964 by Bujard and Moran.[63] More recently, Moughan and Rutherfurd utilised MIU for the analysis of digesta (Section 2.2.1).[38] This method follows a similar pathway to the above reagents, with reaction of MIU with free lysine to give homoarginine after acid hydrolysis (Figure 2.2-h).



o-methylisourea homoarginine

Figure 2.2-h: Reaction of o-methylisourea with protein bound lysine. Adapted from Hurrell et al.[8]

Homoarginine can then be quantified using ion-exchange chromatography or gas chromatography. Results achieved are similar to those obtained by animal tests, but it is unfortunately a time-consuming method, taking 2 – 4 days to complete.[8] Also, low absolute values are obtained, potentially because of side reactions occurring as a result of the long reaction period.[8]

Dye-binding methods

The ability of azo dyes (Figure 2.2-i) to bind to positively charged amino acids (lysine, arginine, histidine) has been used for many years to determine total protein content of samples that have almost constant amino acid composition.[8] This is known as the dye binding capacity method (DBC), as the dye-protein complex precipitates. This precipitate, containing the reacted dye, is filtered from solution and the reduction in the dye concentration of the solution is measured spectrophotometrically.

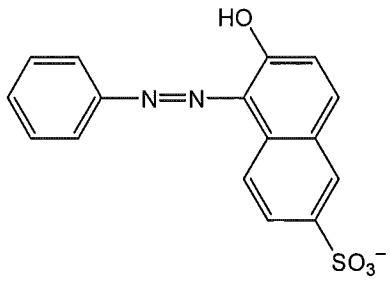


Figure 2.2-i: Structure of Acid Orange 12, an azo dye. Adapted from Hurrell et al.[8]

Hurrell and Carpenter proposed the method of testing for reactive lysine by treating protein with propionic anhydride, to give propionylated lysine, which the azo dyes do not bind to (Figure 2.2-j).[64] Therefore, measuring the difference in dye binding capacity of a solution before and after propionylation, provides a measure of the lysine content of the solution.[65] This is hence termed the differential dye-binding capacity (dDBC) procedure.[65] While this method is feasible for late Maillard reacted products, the azo dyes still react with the basic early Maillard reaction product deoxyketosyl lysine, which also appears to be susceptible to propionylation, hence skewing results.[6,8]

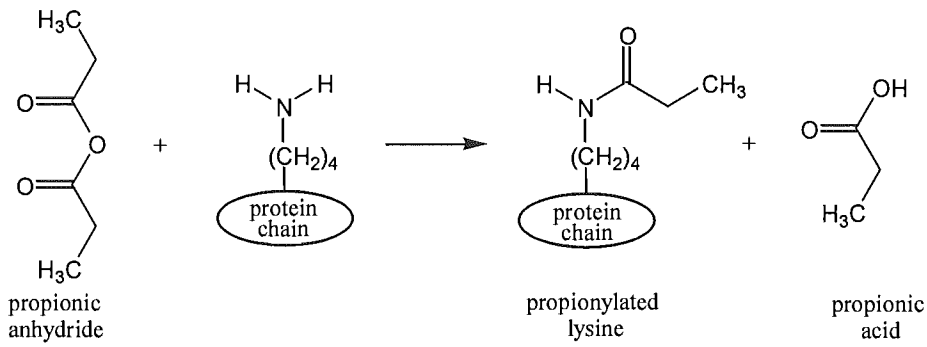


Figure 2.2-j: Propionylation of lysine. Adapted from Hurrell et al.[8]

Ninhydrin method

In the ninhydrin assay, which has been widely utilised in both chemistry and biochemistry,[66] a coloured chromophore results from the reaction of ninhydrin with aliphatic

amines.[67] This chromophore was discovered in 1910 by Ruhemann, and subsequently named Ruhemann's purple (Figure 2.2-k).[68]

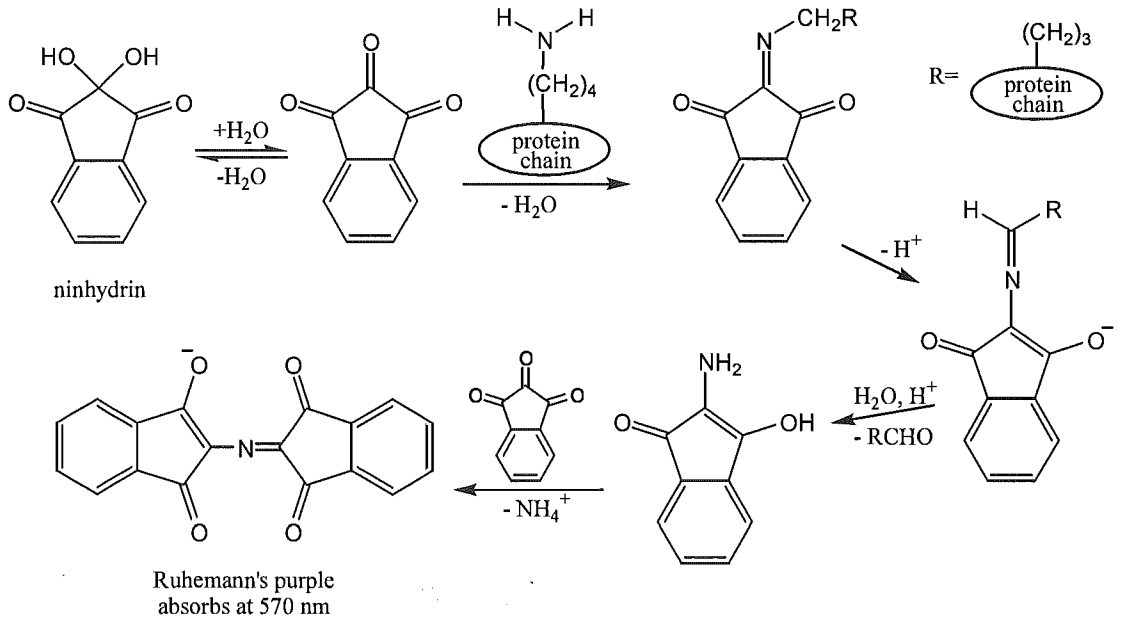


Figure 2.2-k: Reaction of ninhydrin with protein bound lysine. Adapted from Schertz et al.[69]

Friedman *et al.* studied the use of ninhydrin for the determination of lysine in food proteins, and optimised the procedure for these conditions.[70] The ninhydrin method developed involves a one step protein extraction/lysine content assay and uses a hydrindantin/ninhydrin reagent to react with lysine residues, followed by spectrophotometric quantitation of the resulting coloured chromophore. Along with a change in buffer, this method was shown to keep proteins in solution, stabilise the ninhydrin chromophore and give good reproducibility with a range of food proteins.[66,70]

OPA method

Fluorescent chromophores have also been utilised to assay for reactive lysine. *o*-Phthaldialdehyde (OPA) is a fluorescent compound, well known as a reagent for measuring α-amino groups, which is often utilised for precolumn derivitisation of amino acids for amino acid analysis.[71,72] Its use in the fluorometric assay for α-amino groups was

first described by Roth and Hampai.[73] The fluorescence can be quenched by peptide bonds however, and the ability of the compound to absorb light strongly at 340 nm has therefore also been utilised.[74]

The reaction of OPA with amino groups, in the presence of a reducing agent, is shown in Figure 2.2-I. It is the 1-alkylthio-2-substituted isoindole that is the fluorescing and the absorbing compound, although it can undergo further reaction to give the non-fluorescing 2,3-dihydro-1*H*-isoindole-1-one.[75] The OPA method has been shown to have good equivalence with the more established FDNB method in Maillard reacted dairy products, and is inexpensive and quick to perform.[35] However, it cannot be used with proteins that are not water soluble.[35]

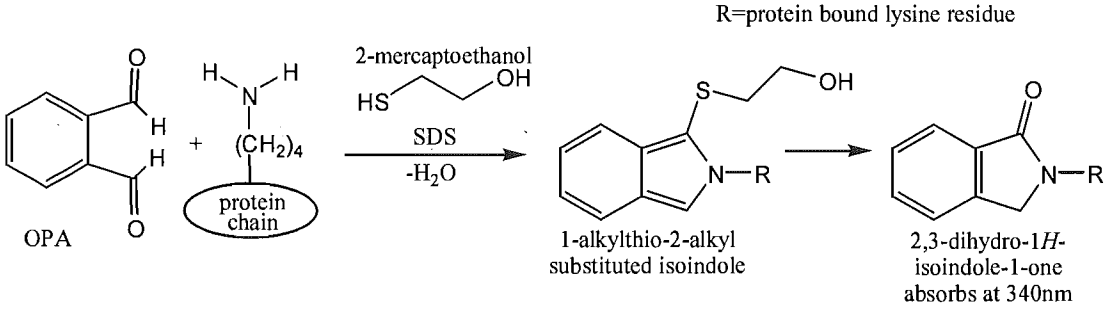


Figure 2.2-I: Reaction of OPA with protein bound lysine. Adapted from Wallace and Fox, and Svêdas et al.[74,75]

Fluorescamine method

Fluorescence is also utilised in the fluorescamine method. Fluorescamine (4-phenylspiro(furan-2(3H),1'-phthalan)-3,3'-dione) is a synthetic compound that was developed in 1972 by Weigele *et al.*[76] Fluorescamine itself is non-fluorescing, but it reacts with primary amines to give a fluorophore that can be assayed at 480 nm (Figure 2.2-m). To give a product on reaction with ε-amino groups, the assay must be carried out at pH 8. Unfortunately, this method has a lower detection limit of around 15% lysine blockage, and interference from fluorescing Maillard reaction products can occur.[77] This method does not appear to have been used extensively for lysine analysis.

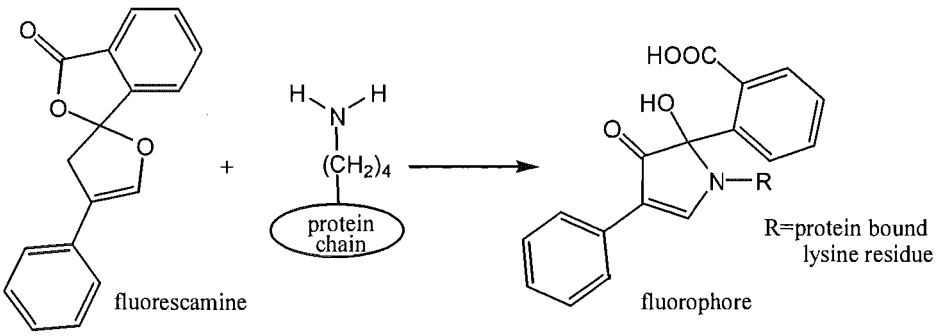


Figure 2.2-m: Reaction of fluorescamine with protein bound lysine. Adapted from Wallace and Fox.[74]

Furosine method

As more researchers have become interested in testing the nutritional quality of food after Maillard reaction has occurred, more methods for testing lysine have been developed specifically for Maillard-reacted foods. One such technique is the furosine method.[78] Furosine was first detected in 1966 by Erbersdobler, and is currently used in the analysis of milk.[79] It is an acid hydrolysis product of fructoselysine, the major Amadori product of Maillard reacted lysine (Figure 2.2-n).

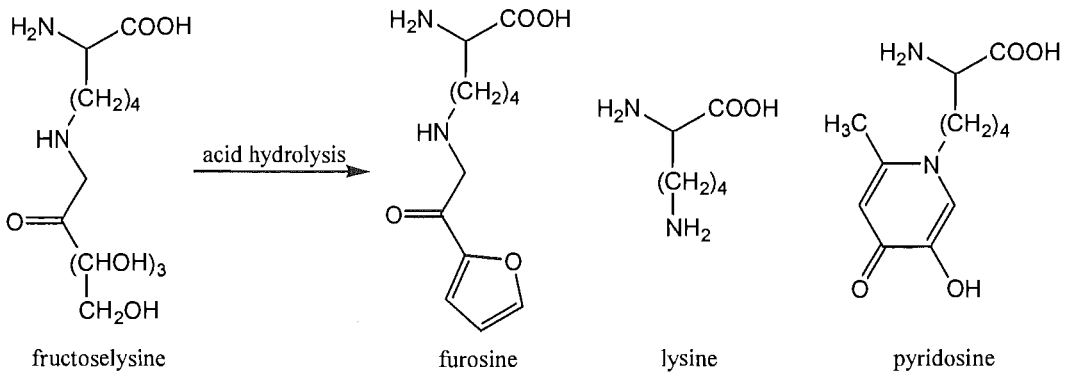


Figure 2.2-n: The major products formed on acid hydrolysis of fructoselysine. Adapted from Erbersdobler.[78]

In the furosine method, the predictable degradation behaviour of fructoselysine during acid hydrolysis is utilised, in which 40% lysine and 32% furosine are generated.[59] The short basic column of the amino acid analyser can be used to easily detect furosine. Therefore,

by measuring the furosine content after acid hydrolysis, a quantification of early Maillard reacted lysine can be made.[77] In combination with total lysine, this can be used to estimate reactive lysine. This method is not suitable after a large amount of late Maillard reaction has occurred, as furosine production reaches a steady-state concentration, while lysine blockage has been shown to continue.[77] It also assumes that all lysine reaction proceeds *via* fructoselysine.[78]

Sodium borohydride

The sodium borohydride method, introduced by Hurrell and Carpenter in 1974, is another method specifically designed for measuring reactive lysine after Maillard reaction.[80] In this assay, sodium borohydride reduces deoxyketosyl lysine to a form that does not give lysine or furosine after acid hydrolysis (Figure 2.2-o).

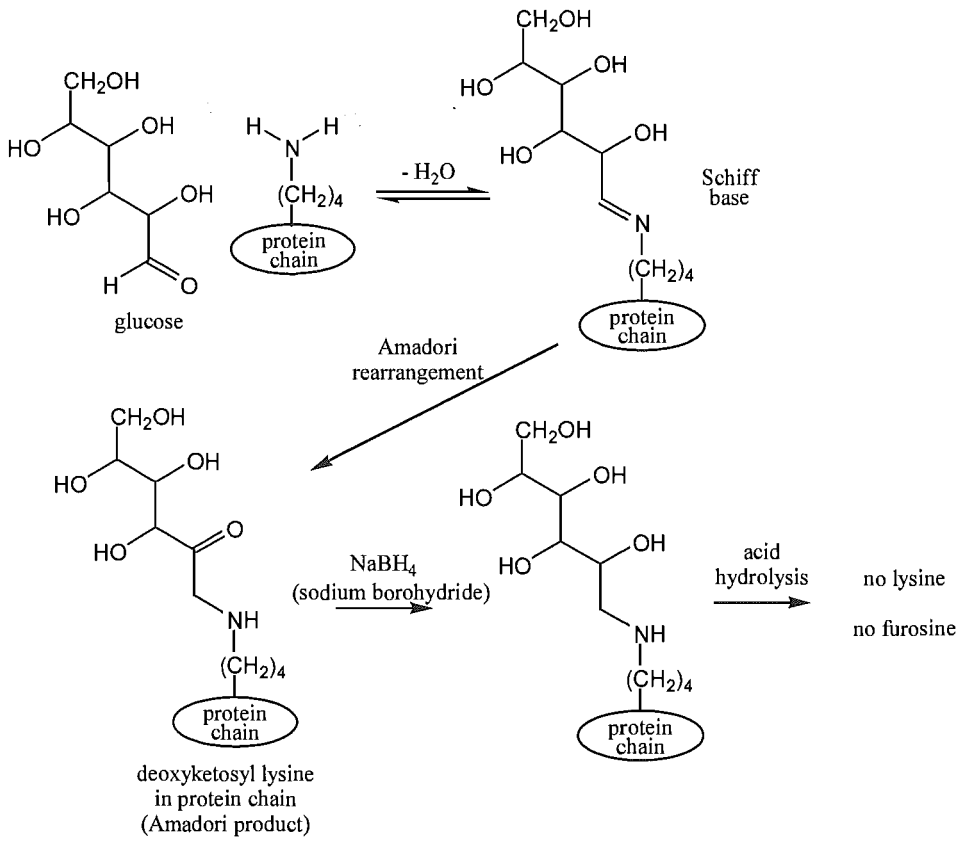


Figure 2.2-o: Deoxyketosyl lysine is produced by the Maillard reaction of glucose and lysine, through rearrangement of the Schiff base. Reaction of the deoxyketosyl lysine product with sodium borohydride gives a product that does not form lysine or furosine on acid hydrolysis. Adapted from Hurrell et al.[8]

Hence, on acid hydrolysis, the total amount of lysine released was assumed to be a measure of those lysine residues that had not undergone Maillard reaction. It appears, however, that sodium borohydride also reduces the biologically available Schiff base form of lysine.[8] As Schiff bases exist in equilibrium with "lysine + carbonyl containing group" this reduction could result in the formation of more Schiff bases.

Alternative methods

An alternative approach to the above *in vitro* methods is to mimic the processes occurring to the protein in the animal and then measure for free lysine. This approach, such as that developed by Mauron in 1970, involves replicating the processes occurring in the digestive system.[81] Mauron developed an enzymatic assay using pepsin with pancreatin, and dialysis during hydrolysis.[81] Results indicated that the proportion of lysine released agreed well with a rat assay and FDNB-reactive lysine. In another assay, developed by Raynor and Fox, pronase was used, and good agreement was found between lysine released and the FDNB-difference method.[59]

These methods use proteolytic enzymes, which only release reactive lysine, unlike acid hydrolysis. However, they can only be used to compare lysine contents, as opposed to giving absolute values, as enzymatic hydrolysis is never complete and only free lysine in the hydrolysate is measured.[59] Reactive lysine may still be present in small peptides, but is not measured as acid hydrolysis cannot be used to break down the peptide, as this would also release non-reactive lysine.[59]

Enzymes are also utilised in biosensors, a potentially fast and accurate method of measuring lysine content of food systems such as milk.[82] However, these techniques tend to have low specificity for lysine, with interference as large as 80% from other amino acids such as arginine.[82] A method has been developed by O'Connell *et al.* that utilises a sensor that detects hydrogen peroxide produced by the enzymatic oxidation of L-lysine after microwave acid hydrolysis.[82] As acid hydrolysis is involved, this technique is unlikely to differentiate between acid labile early Maillard reaction products and lysine.

An approach similar to mimicking digestive processes is to utilise microbes. Microbiological assays have been developed for the determination of lysine content, but

when Maillard reacted proteins have been used results have not been ideal.[59] Unfortunately, some microbes can utilise early Maillard reaction products such as ε-fructosyl-lysine, giving results that are inaccurate for animals.[59] However, a microbial assay utilising a luminescent, lysine-auxotrophic strain of *Escherichia coli* has recently been developed, and shown to have potential for testing heat-damaged proteins.[83,84]

2.2.4 Method comparison

Given the number of methods available for measuring the lysine content, choosing the best method for a particular situation can be a challenging task. A number of studies have been carried out comparing most of the chemical methods available.[4,8,85] These studies have looked at both the potential of methods to measure the lysine contents of samples, along with the ability to measure the relative amount of lysine lost through processing.

In Figure 2.2-p, data are presented from a study by Hurrell and Carpenter, where various methods are compared for their ability to give true values for lysine contents in an unheated albumin/glucose sample. Compared with growth assays, all tests slightly underestimated lysine contents, and both the TNBS and the MIU methods did so significantly.[86]

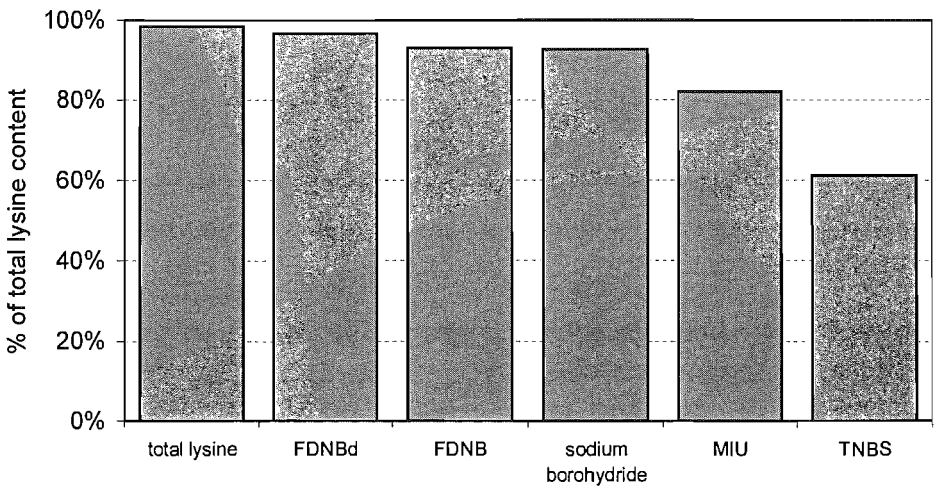


Figure 2.2-p: Comparison of the ability of various methods to give a true value for the level of lysine in an unheated albumin control sample. Data are presented as percentage difference from the value found from rat and/or chick growth assays. Adapted from data presented in Hurrell and Carpenter.[8]

In Figure 2.2-q, the results of two studies by Hurrell and Carpenter are shown, where the ability of various methods to estimate lysine loss through processing is examined. From these results, it is apparent that the FDNB method gave the overall best comparison with the growth assays, under the conditions used. The sodium borohydride method also gave reasonably close approximations, but did tend to underestimate bioavailable lysine, which can be explained by the propensity of this method to give a negative result for the biologically available Schiff base. The dDBC method closely approximated growth under the conditions it was used in, as did the MIU method.

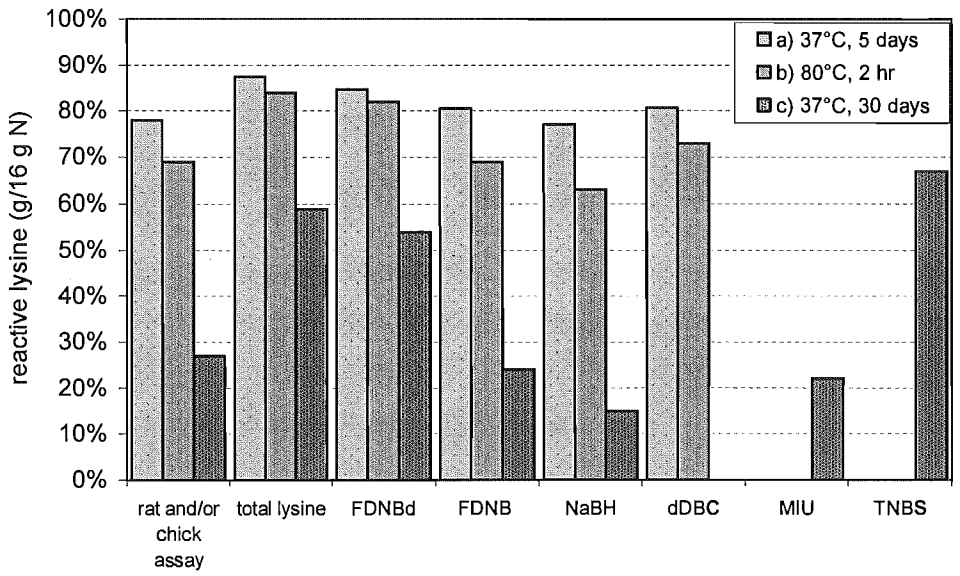


Figure 2.2-q: Comparison between different techniques for measuring lysine in albumin-glucose mixtures, for samples heated under different regimes. Adapted from data in Hurrell and Carpenter (1978) (a and b);[85] and from Hurrell and Carpenter (1981) (c).[8] (No data available for dDBC, condition c; MIU, TNBS conditions a and b.)

In contrast, amino acid analysis (total lysine) and the FDNBd method overestimated bioavailable lysine in all conditions. The TNBS method, which was only used in condition c, also strongly overestimated bioavailable lysine. The conclusion from Hurrell and Carpenter was that the direct FDNB, $NaBH_4$, and MIU methods were the most suitable, of the methods tested, for measuring lysine after early Maillard reactions.[8] It was noted that the furosine method could also be included if the sample to be tested was milk powder.[8]

Subsequently, in the literature, studies have compared the FDNB method with the MIU method adapted by Moughan and Rutherfurd,[38] the furosine method, and with the OPA method.[35] Both of these studies showed good comparison between FDNB analysis, and the methodology under investigation. The study by Vigo *et al.* showed no significant difference between the OPA method, with no dialysis, and the FDNB method, with dialysis, in sugar containing systems under various heating regimes, indicating that the OPA method, which is quicker and cheaper than the FDNB method, can give equivalent results.[35]

The OPA method was selected for much of the work in this thesis, as the most appropriate for assaying for reactive lysine in model systems. It fulfils the criteria of being convenient, rapid and cheap to perform. It has also been shown in previous research to be suitable for Maillard reacted systems.[35] Unlike the FDNB and TNBS methods, the presence of sugars does not appear to interfere with the assay,[35] and the method does not involve acid hydrolysis, limiting the production of artefacts. The ninhydrin method, as developed by Friedman *et al.*,[70] was selected for use in flour systems, where the OPA method was reported not to be appropriate, due to the low solubility of some flour proteins. This unsuitability was supported by experiments described in section 2.5.3, where the precipitation of barley flour proteins during analysis *via* the standard OPA method prevented spectrophotometric quantitation. The reaction of flour proteins are investigated in chapter 4, where they are used in a system modelling pelleting.

2.2.5 Crosslinking during the Maillard reaction

The formation of crosslinks as a result of the Maillard reaction is well established, as discussed in chapter 1.[87] Crosslink formation is of nutritional relevance, as unreacted lysine residues may not be bioavailable if the protein is crosslinked in such a way that digestive enzymes cannot release it. The formation of crosslinked products is important in many research fields and, in addition to lysine loss, crosslinking behaviour was also relevant for this thesis, as a further tool for investigating Maillard reaction in protein systems. For relatively small pure proteins, the progression of crosslinking behaviour under Maillard reaction conditions can be analysed using sodium dodecyl sulphate-

polyacrylamide gel electrophoresis (SDS-PAGE), as shown by Miller *et al.*[88] This methodology shows the macromolecular effects of the Maillard reaction, particularly crosslinking and the formation of protein breakdown products. Size-exclusion HPLC is also a useful method for assessing the crosslinking reaction of proteins,[89] and is not limited to proteins with a low molecular weight. However, it is more time consuming and expensive than SDS-PAGE.

2.3 Following the Maillard reaction using pH

During the Maillard reaction, a decrease in pH occurs.[90] This decrease in pH was followed in model systems undergoing Maillard reaction, by monitoring the pH. Equimolar solutions of lysine (pH 7) and carbohydrate (pH 7) were mixed, and the change in pH was followed for 20 hours, with samples maintained at 37°C.

Initial results with simple carbonyl compounds (section 1.5.2) appeared promising, with the pH drop for diacetyl and glyoxal greater than those for glucose, xylose and cyclotene, as may be expected from previous results from crosslinking.[88,91] However, a significant drop in pH was seen in some carbohydrate controls, which was attributed to carbon dioxide dissolving into solution, as similar pH changes were seen when water alone was monitored (Figure 2.3-a). No drop was seen in the lysine control, presumably due to the buffering action of lysine. As control and reaction sample pH profiles fitted within those seen in the Figure 2.3-a, it was apparent this was not an appropriate method with which to follow the Maillard reaction under the reaction conditions of this study.

As a result of the above findings, a pH stat method was investigated. This method was very similar to that shown above, but sodium hydroxide was added by a pH stat machine to keep the pH at 7 throughout the experiment. Results suggested that this methodology is more appropriate than monitoring pH change for the investigation of the Maillard reaction. For both glyoxal and diacetyl, the reaction mixture required a significantly greater volume of base, compared with the controls, to maintain a pH of 7 (glyoxal results shown in Figure 2.3-b).

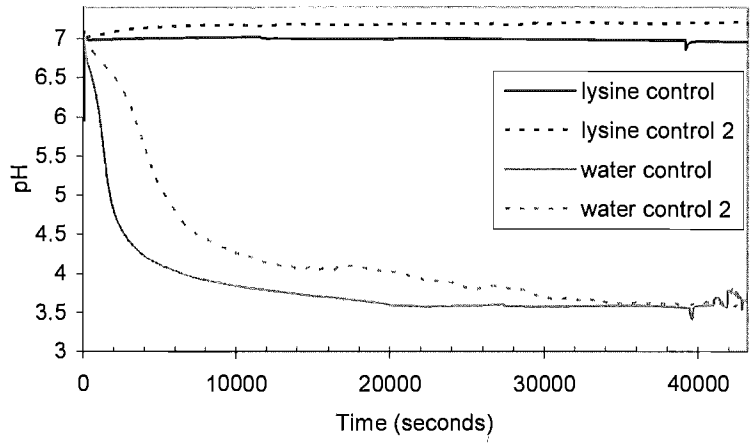


Figure 2.3-a: pH profiles of control solutions consisting of distilled water or distilled water and 137 mM lysine, at 37°C. Shown for the first 12 hour period.

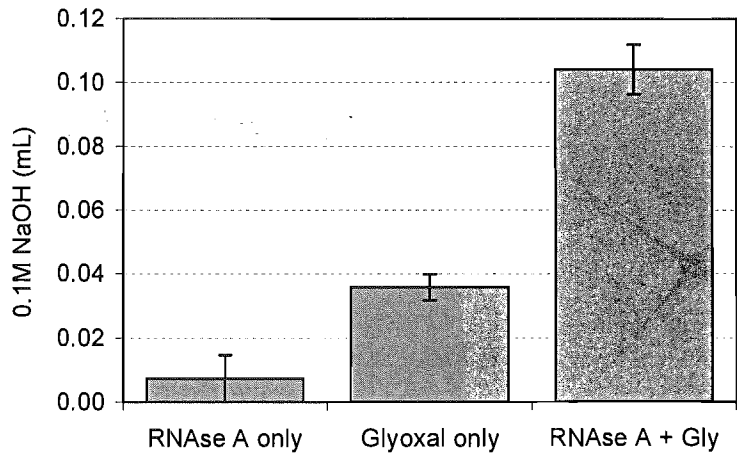


Figure 2.3-b: Comparison of volume of 0.1 M sodium hydroxide required to keep a 1 mL control and reaction solutions of RNase A and glyoxal at pH 7 for 12 hours at 37°C. Error bars represent standard error of three replicates.

Unfortunately, difficulties were encountered after a few hours in some experiments, with pH values becoming erratic at points where no base was being added. This was possibly due to the low volumes used, with evaporation making the level for the pH probe too low. Taking this into account, along with the high cost of RNase A for this experiment, it was decided to follow the Maillard reaction using lysine counts and crosslinking studies and not pH. However, this methodology has potential for use in studying lysine and carbohydrate model studies over short periods of time.

2.4 METHOD SELECTION AND DEVELOPMENT – STUDYING THE PROGRESSION OF THE MAILLARD REACTION IN MODEL SYSTEMS

In the study of the Maillard reaction in model systems, the main analysis tools used were SDS-PAGE, as discussed in section 2.2.5, and the OPA and ninhydrin methods for the quantification of the free amino groups, as discussed in section 2.2.4. These methods were developed in order to make them appropriate for both simple and more complex model systems, as well as testing of actual feed pellets.

2.4.1 Selection of model system

Ribonuclease A (RNase A) was chosen as a model protein as it is readily available commercially, and is relatively stable, both thermally and chemically.[92] RNase A is ideal for crosslinking studies, as the oligomerisation of the monomer is easy to detect by SDS-PAGE, due to the small monomer size (13,682 Da). RNase A contains eleven free amino groups, ten as ε-amino groups of lysine residues, and the other being the α-amino group of the N-terminal end of the protein. While RNase A is not highly similar to proteins found in chicken feed, the size of flour proteins limit their utility in model studies where crosslinking is under investigation. Therefore, to understand at a basic level the reaction of lysine residues in pure, well controlled conditions, it was decided that RNase A was a suitable protein to use.

For model studies in systems closer to that of chicken feed, barley flour was chosen. Barley is an important constituent of chicken diets in the South Island of New Zealand, due to local availability. Protein extraction was performed according to the standard method described in Marchylo and Kruger.[93] The experiment described in section 2.5.3 indicated that this extraction method does not affect lysine residues.

2.4.2 Measuring RNase A concentration

In order to give accurate comparisons between lysine contents in model studies, a method of measuring protein concentration was required that was not significantly perturbed by

Maillard derivitisation. The Bradford method was chosen as an established, simple, inexpensive method that is relatively sensitive.[94] Calibration curves with RNase A were established (Figure 2.4-a), using the micro version of the Bio-Rad Protein assay, which utilises the Bradford methodology.[95] This showed the linear range of the Bradford method with RNase A to be between approximately 1 and 11 µg/mL RNase A. However, as can be seen from Figure 2.4-a, the slope of the calibration is very low, with a gain of only 0.009 absorbance units for every µg/mL increase in RNase A (Figure 2.4-a: standard). This means that small errors could have significant effects on the results of protein concentration measurement.

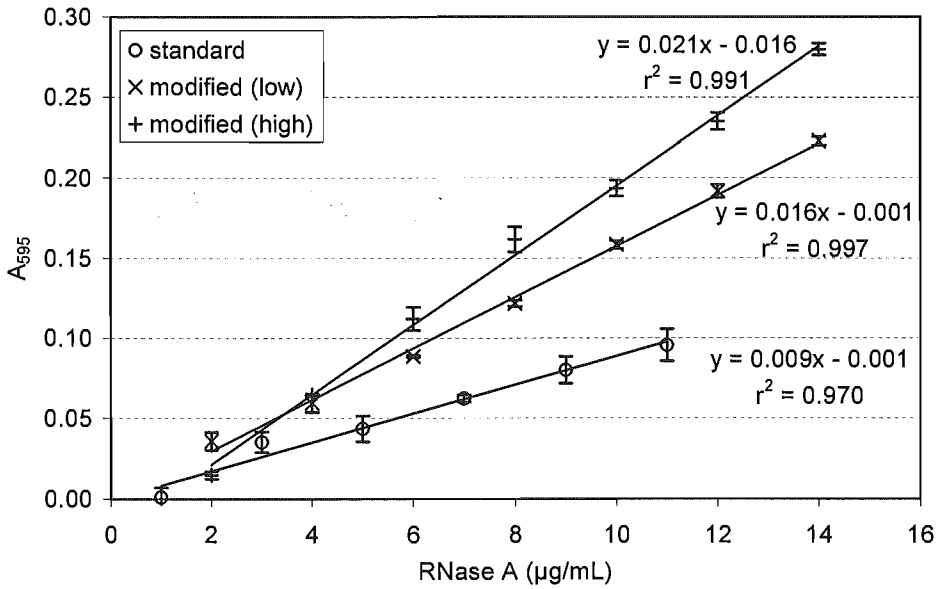


Figure 2.4-a: Calibration curves for the Bradford method with RNase A. Curves are shown for the standard method and for the modified method (data for high and low response batches of RNase A shown). Error bars represent the standard error of two calibrations with triplicate readings performed in each for the standard method, and standard error of triplicate readings for the modified method calibrations.

To overcome this issue, a modification suggested by Duhamel *et al.* was tested.[96] Duhamel *et al.* added sub-threshold levels of SDS to samples containing collagen, as collagen gives a similarly low response with the Bradford assay. SDS interferes with the assay,[97] but at sub-threshold levels, where SDS by itself showed no absorbance,

improvement in the sensitivity of the assay to collagen was seen.[96] Therefore, this technique was trialled using RNase A.

The modified assay gave improved response curves, as seen in Figure 2.4-a, with slopes between 1.7 and 2.4 times greater than the standard method and an extended linear range. However, the response was still relatively low. This shallow calibration curve associated with testing RNase A concentration, even after method modification, exacerbates the complexity involved in quantifying lysine content accurately. The range in slopes for the modified method is indicative of the difference between batches of RNase A, discussed further in section 2.5.1.

2.4.3 Reaction of RNase A with carbohydrates

For the study of the Maillard reaction in model systems, a variety of carbohydrates were reacted with the RNase A to investigate the effect of a variety of relevant carbohydrates on the Maillard reaction. Many challenges were encountered when trying to obtain results from these analyses. Issues of result replication and measurement of meaningful amino group concentrations were the main difficulties experienced.

Carbohydrate

The carbohydrates studied included cyclotene, xylose, glucose and sucrose as pure, well defined carbohydrates,[98,99] and dextrin, starch, molasses and malt extract as carbohydrate sources similar to those found in chicken feed.[100] These carbohydrates are further detailed in chapter 3. The methods used to study the progression of the Maillard reaction in models systems had to be suitable for use with all of these carbohydrate types.

Variation between carbohydrate batches led to differences in the degree of reaction observed. General problems are illustrated here for specific sugars, sucrose and glucose. Sucrose is not a reducing sugar, and hence no reaction was expected when it was in the presence of protein. However, under acidic conditions or high temperature, sucrose is hydrolysed to the reducing glucose and fructose, which accounts for variations seen in incubations with sucrose.[101] Glucose gave the clearest indication of inter-batch variation,

with one batch showing no crosslinking at 50°C after twelve days, and only 12% lysine reaction, compared to the control, and another batch, which showed a 27% reduction in reactive lysine after 2 days at 50°C, and a 79% loss after 8 days. This lack of reaction conflicts with literature reports of incubations of glucose with various proteins examined in the literature, where around 40% of lysine had reacted in proteins incubated at about 37°C for two days, and around 70% at 50°C (Table 2.4-a).

Protein	Temperature	Time incubated	pH	Lysine remaining (approximate)	Reference
RNase A	50°C	8 days	n/a	21%	This thesis
RNase A	50°C	12 days	7[n]	88%	This thesis
bovine serum albumin[a]	50°C	2 days	7[n]	70%	Yeboah *et al.*, 1999.[102]
myosin[a]	30°C 35°C 40°C	50 hours 50 hours 50 hours	7.5[b] 7.5[b] 7.5[b]	90% 70% 60%	Tanabe *et al.*, 2001.[103]
casein*	37°C	10 days	n/a	61%	Smith *et al.*, 1984.[101]

n/a – not measured or altered
[a] dry samples heated, 65% humidity
[n] adjusted to pH 7 before incubation but not buffered
[b] buffered

Table 2.4-a: Percentage lysine remaining after incubation of various proteins with glucose; from literature studies.

This inherent variation in reaction of protein with pure, commercially obtained sugars suggests that the reaction is very sensitive to conditions, such as temperature and particularly pH, and made experiments with feed grade carbohydrates, where precise composition is often unknown, very challenging.

Dialysis tubing

Difficulties with replication of experiments, which included a dialysis step, were encountered. When using one batch of dialysis tubing (molecular weight cut-off 12 kDa), virtually no RNase A (MW 13.7 kDa) was retained within the tubing, whilst other batches retained significant amounts of RNase A within the tubing. This may have been due to

either variation in pore size or fragmentation of the RNase A in some batches, such that all fragments were less than the molecular weight cut-off of the dialysis tubing. It is also possible that a batch of dialysis tubing had been mislabelled by the suppliers. Thus careful measurement and controls were essential at every step to ensure meaningful measurement.

2.5 METHOD DEVELOPMENT – LYSINE TESTING IN MODEL SYSTEMS

2.5.1 Quantification of lysine in RNase A *via* the OPA method

As discussed in section 2.2.4, the OPA method was chosen as the most suitable for testing lysine in RNase A model systems. Calibration curves using lysine (Figure 2.5-a) and RNase A (Figure 2.5-b) were carried out. RNase A gave consistently straight calibration curves over a wide concentration range, with an r^2 of 0.998 for six calibrations. However, the slope of the calibrations did vary considerably with different batches of protein, with the greatest slope almost 1.5 times greater than the least, as can be seen in Figure 2.5-b. Thus new calibration curves were prepared with each batch of protein.

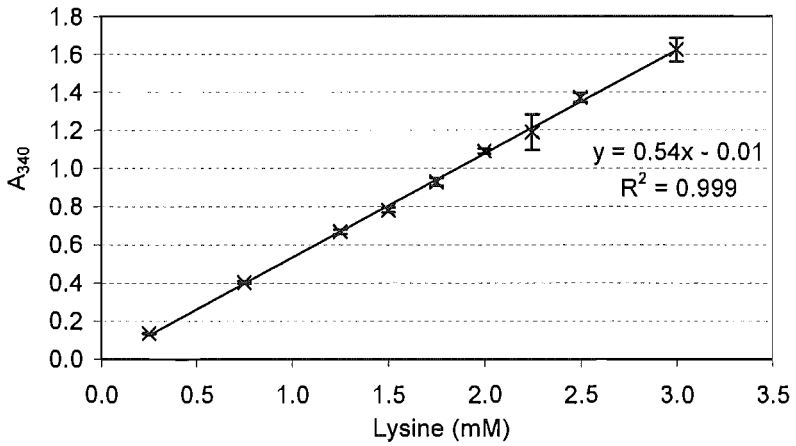


Figure 2.5-a: Calibration curve for the OPA method using lysine as a standard. Average of twelve calibrations shown, with triplicate readings performed for each data point in each calibration. Error bars represent standard error of replicate calibrations. Not all calibrations were performed at each concentration level.

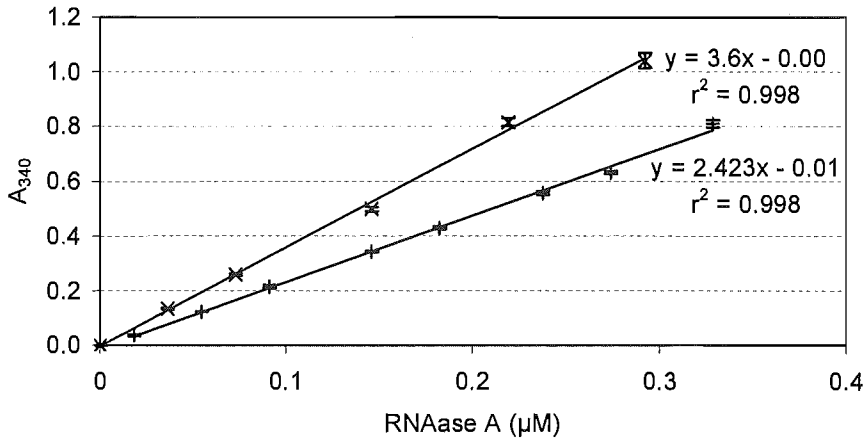


Figure 2.5-b: Calibration curves for the OPA method using RNase A as a standard. Curves shown for the calibrations giving the lowest and highest slope of the correlation. (All measurements performed in triplicate. Error bars represent standard error of the measurements.)

All RNase A was obtained from Sigma; however, different batches did show some variation. In addition to the variation in slope seen for the OPA assay, this batch-wise difference was observed in the varying amount of dimer and lower molecular weight fractions visualised by SDS-PAGE. The propensity of RNase A to form oligimers has been noted in the literature, where Gotte *et al.* observed the formation of domain swapped dimers, trimers and tetramers after heating RNase A in solution.[104,105] Some batches of protein tended to precipitate upon freezing after incubation. Controls were always included in model studies in an attempt to minimise any variation that occurred.

If the calibration curves of OPA with RNase A are compared with those of OPA with lysine, it is possible to estimate the number of amino groups available to react in each RNase A molecule. These calculations gave averages of 8.9 and 13.3 amino groups per RNase A molecule in the calibrations with the least and greatest slope, respectively. As noted above, there are only 11 amino groups in each RNase A molecule. This would indicate that, in batches giving a steeper slope, the RNase A may have fragmented, leading to an increase in terminal amino groups available to react with OPA. This is consistent with results found from SDS-PAGE, where smudged bands at low molecular weight were

apparent for the frozen control, a sample containing RNase A in water, as seen in Figure 2.5-c.

The presence of 8.9 amino groups able to react with OPA in the lowest calibration indicates little fragmentation had occurred in this sample of RNase A, and that two of the amino groups were hidden within the molecule and unable to react, probable due to steric considerations. Therefore, the total unfolding of RNase A, and hence complete exposure of all amino groups, that may have been expected given the presence of a reducing agent and SDS in the assay solution was not observed. However, this was consistent with previous research that indicated that the secondary structure of RNase A remains stable in conditions such as those used in the OPA assay.[106]

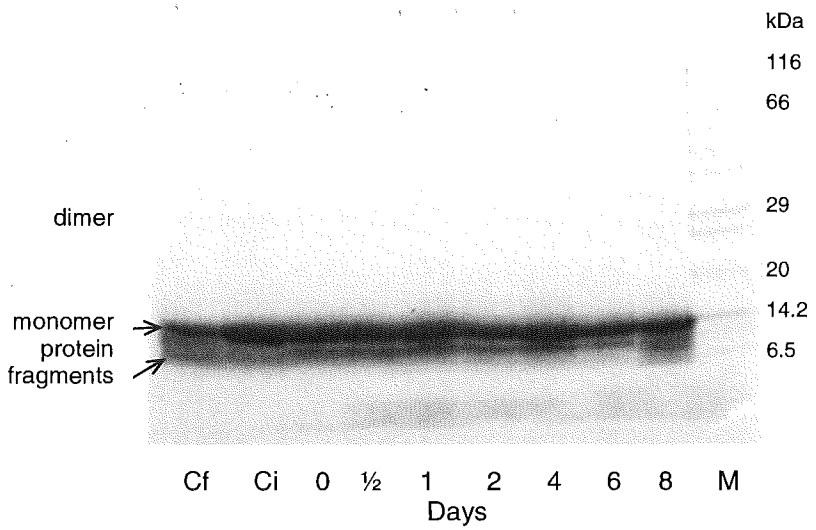


Figure 2.5-c: Typical SDS-PAGE gel of 25 mg/mL (1.8 mM) RNAse A in aqueous solution, showing the presence of small molecular weight protein fragments in the frozen control sample. Samples incubated with 25 mg/mL dextrin at 37°C, pH7, for 0-8 days. Cf: Frozen control; Ci: incubated control (RNAse A only); M: Sigmamarker (wide-range).

This indicates that even purified proteins, known for stability, can have significant inter-batch variations. In addition, this highlights the difficulties associated with protein fragmentation for all lysine testing methods that are dependent on the reaction of free amino groups. Therefore, it was important to maintain consistency of RNase A batch within each experimental set, and calibrate lysine assays accordingly.

Interference

While literature reports that the OPA lysine assay is not affected by carbohydrate interference,[35] positive interference was seen when using some carbohydrates in these model systems. This was taken into account by using carbohydrate controls. It is also possible that Maillard reaction products could interfere with the assay. It was assumed this interference was not significant.

2.5.2 Quantification of lysine in barley flour *via* the ninhydrin method

Since flour proteins are not aqueous soluble, from dry flour, the ninhydrin method was chosen for use in the more complex flour systems. Barley proteins, like most grain proteins, are of high molecular weight and have low water solubility. Friedman *et al.* have shown the utility of the ninhydrin method in a number of food systems, including barley flour.[66,70] Calibration curves for leucine were established, and showed good linearity with an r^2 = 0.996 (Figure 2.5-d). Leucine was chosen as a standard because the molar absorptivity of the ninhydrin chromophore derived from it is identical to authentic Ruhemann's purple.[70]

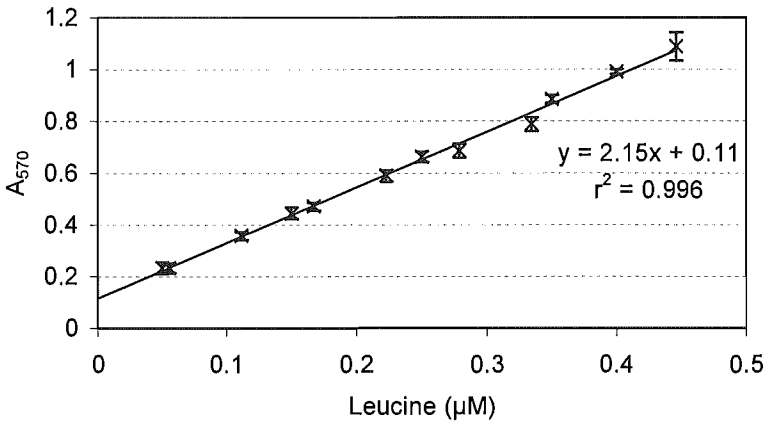


Figure 2.5-d: Calibration curve for the ninhydrin method using leucine as the standard. Average of six calibrations shown, with duplicate readings performed for each data point in each calibration. Error bars represent standard error of replicate calibrations. Not all calibrations were performed at each concentration level.

It has been demonstrated that a correction factor is required for comparison between the leucine standard and proteins. Using this method, Friedman *et al.* showed proteins to have leucine equivalents per lysine residue of between 0.638 (lactalbumin) and 1.10 (bovine serum albumin).[70] These were calculated using the equations below.

$$\text{leu equivalent per lys residue} = \frac{\text{observed absorbance}}{\text{theoretical absorbance}}$$

$$\text{theoretical absorbance} = \frac{(\text{molar absorptivity of leu})(\text{moles lys/100g sample})}{\text{final volume of the ninhydrin solution x 100}}$$

The equivalence value for wheat flour, calculated using data given by Friedman *et al.* and using the above equations without adjustment for terminal amino groups, was 1.78.[70] Therefore, approximately half the contribution to the ninhydrin absorbance was from N-terminal amino groups. Similarly, the equivalence value for barley flour was calculated from the data of Friedman *et al.*, to give a value of 1.49. This value was used as the adjustment, as shown below, for investigations using the ninhydrin method to test barley flours in section 2.5.3, and for testing the lysine content of barley flour model systems in chapter 4.

$$\text{reactive lysine content} = \frac{\text{ninhydrin reactive amino groups}}{1.49}$$

Difficulties were encountered with this ninhydrin method when assaying flour samples that had free lysine added. This was due to the small amount of flour required for the ninhydrin assay, of around 6 mg, for samples without added free lysine. With free lysine added, sample homogeneity became exceedingly important, and large dilutions were required once the ninhydrin reaction was complete. Therefore, it was decided that a modification of the OPA method, to make it suitable for use with flour proteins, would be attempted. This would enable larger amounts of flour, from 50-100 mg, to be weighed out for subsequent protein extraction. Thus, complete homogeneity of the flour was less essential. If required, dilution of the extracted protein could be performed prior to analysis by the modified OPA (mOPA) method.

2.5.3 Development of the OPA method to enable quantification of lysine in a barley flour system

Despite having several advantages compared with other chemical procedures, such as being technically simple, rapid to perform, having no requirement of severe heat treatment or hydrolysis, having a lack of interference from sugars and a requirement for only small quantities of sample, the published OPA method cannot be used for proteins which are not soluble.[35] This modification overcomes this problem for barley flour systems, and potentially other insoluble protein systems also.

Barley was used as a standard grain for the development and assessment of the modification of the OPA method. The standard total protein extraction method for barley flours utilises a 1% dithiothreitol, 50% 1-propanol solution, with heating to 60°C for 30 minutes.[93] The same solution can be used in the total extraction of wheat proteins.[107] Hence, the OPA method was adapted so that the final solution contained 50% 1-propanol, the standard reagents: SDS, OPA, methanol, and β-mercaptoethanol; with the balance of the volume made up using the standard buffer of 0.1 M sodium borate. When first trialled, a white precipitate formed. It was determined that this precipitation was due to the insolubility of the sodium borate buffer in 50% propanol, so an alternate buffer was required. A bicine buffer was chosen, due to its less ionic nature and an appropriate buffering pH range.[108] The pH of this bicine buffer was adjusted to 9.4 so as to be equivalent to that of 0.1 M sodium borate, as used in the unmodified method.[108]

Analysis of this method with lysine (Figure 2.5-e) gave results that were similar to those obtained in the standard OPA method. It was therefore concluded that the mOPA method was valid for measuring lysine concentration in model systems.

In order to determine the validity of the mOPA method in flour systems, 36 barley flours (4 cultivars grown at 9 sites) were tested for lysine content by the mOPA method, the ninhydrin method and by amino acid analysis.

The mOPA figure was adjusted for N-terminal amino groups as suggested by Friedman (section 2.5.2).[70] In Figure 2.5-f the average lysine content of the thirty-six flours, as measured by the three different methods, are shown. In food where no Maillard damage had occurred, the values for lysine content found by ninhydrin and mOPA analysis should

equal those found by amino acid analysis. This indicates that some Maillard reaction had occurred in the flour samples during milling or storage, given the 12% lower values for the mOPA and ninhydrin measurements as compared with the value found from amino acid analysis.

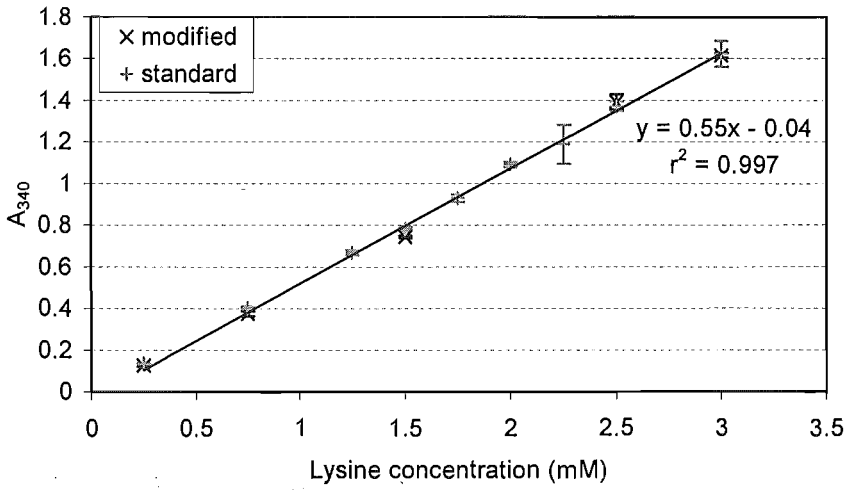


Figure 2.5-e: Calibration curve for the mOPA method using lysine as a standard, as compared with the calibration curve for the standard OPA method. Points represent mean of triplicate readings. Error bars represent standard error of triplicate readings.

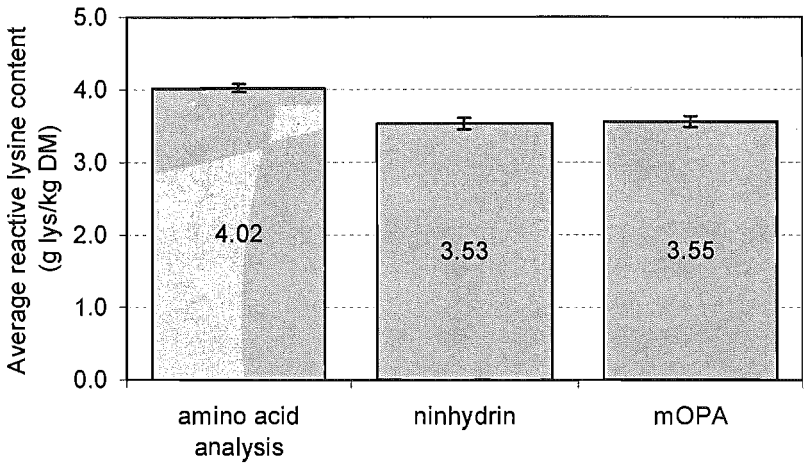


Figure 2.5-f: Comparison of the average lysine content for the 36 barley flours as tested by three different methods. Error bars represent the standard error around the average lysine content of the 36 flours.

The mOPA method correlated well with the ninhydrin method, with an r^2 of 0.85. In Figure 2.5-g, the lysine contents found by mOPA method and ninhydrin method are correlated with values from amino acid analysis. Both methods correlated poorly, indicating that these flour samples had undergone early Maillard reaction, probably as a result of storage or milling, as any acid labile Maillard reacted products would be converted into lysine on acid hydrolysis during amino acid analysis. These same products did not react with ninhydrin or mOPA.

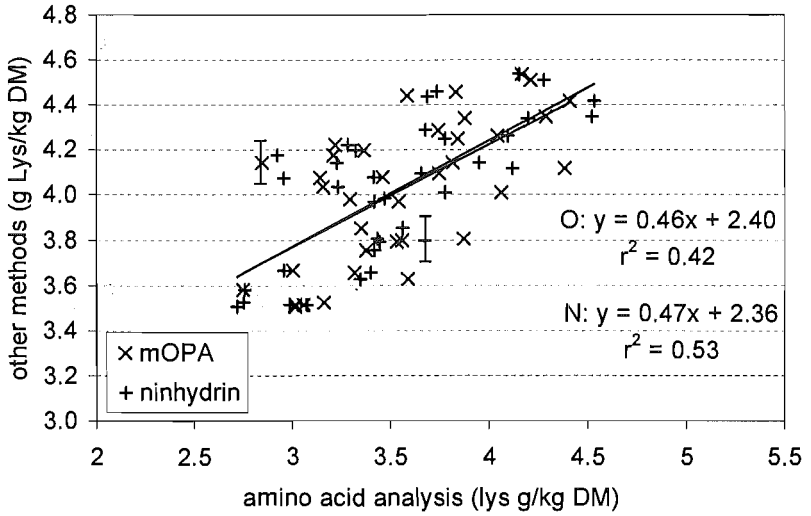


Figure 2.5-g: Comparison of amino acid analysis with O: the mOPA method; and N: the ninhydrin method, for measuring the lysine content of barley flour. Error bars represent the average standard error of triplicate readings on four replicate extractions for the mOPA method and duplicate readings on three replicate assays for the ninhydrin method.

For each flour sample, the reactive lysine content, determined by the mOPA or ninhydrin method, was subtracted from the total lysine content, determined by amino acid analysis. This gave a measure of unreactive, or blocked lysine for each flour sample, as shown in Figure 2.5-h. From this it was determined that the two methods correlated well for the amount of blocked lysine that they measured for each flour sample. Interestingly, values of blocked lysine did not relate to total lysine content of the flour, as can be seen from Figure 2.5-i.

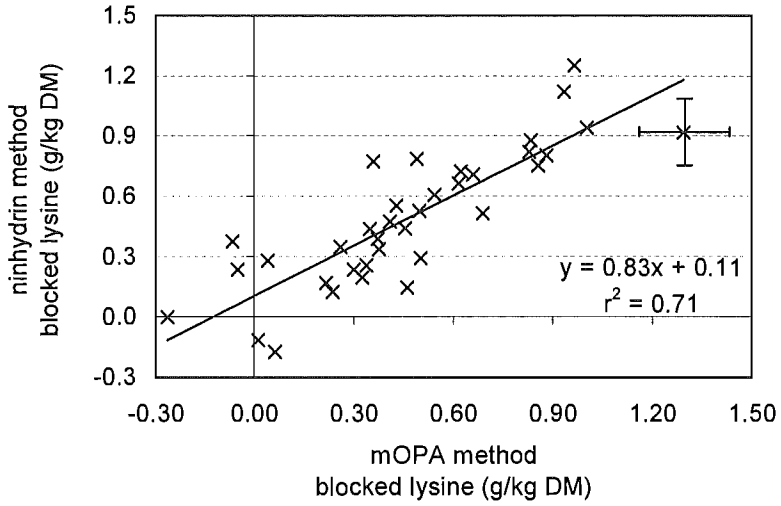


Figure 2.5-h: Correlation between blocked lysine as measured by the mOPA method and the ninhydrin method. Error bars represent the average standard error of triplicate readings on four replicate extractions for the mOPA method and duplicate readings on three replicate assays for the ninhydrin method.

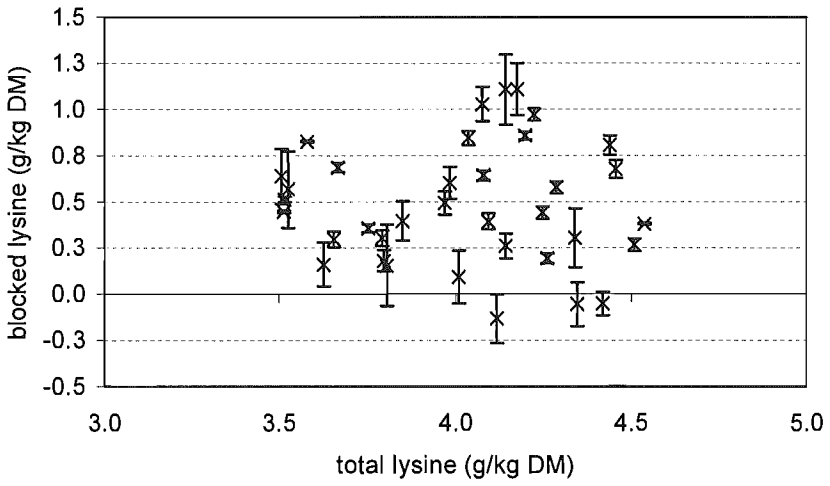


Figure 2.5-i: Comparison between total lysine content of 36 flours, found from amino acid analysis, and blocked lysine (averaged from values gained by ninhydrin and mOPA methods). Error bars represent standard error of two measurement methods.

Analysis of lysine content of 36 barley flours

The 36 barley flours were of 4 different varieties, grown at 9 different sites. Therefore, it was determined whether either of these factors had an affect on the blocked lysine content of the flour. For each of the methods, the blocked lysine contents of the flours from each of the four flour cultivars were averaged. Similarly, the blocked lysine contents of the flours grown at each of the nine sites were averaged. The results from these calculations (Figure 2.5-j) show that while cultivar of flour had little effect on blocked lysine content, site of growth had a large effect.

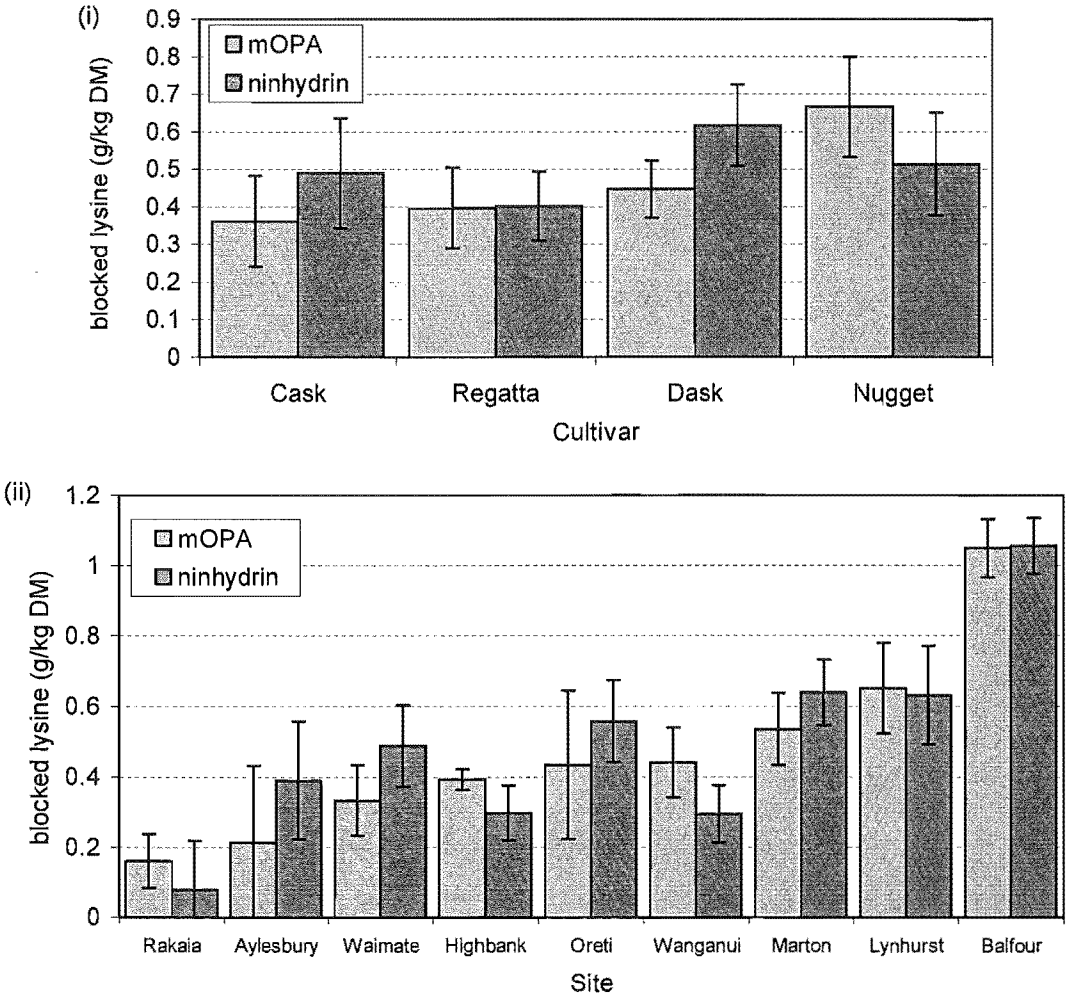


Figure 2.5-j: Effect of (i) cultivar and (ii) site on the blocked lysine content of barley flours, as measured by the mOPA method and the ninhydrin method. Error bars represent standard error of (i) 9 flours and (ii) 4 flours.

Barley grown in Balfour had almost twice as much blocked lysine, as measured by both methods, than barley grown at any other site. This may indicate that somewhere during the history (harvesting, transporting, milling or storage) of the Balfour barley, it was subjected to higher temperatures, and hence a greater degree of Maillard reaction, than barley from the other sites. Unfortunately, as these samples were not under specific investigation for Maillard reaction at the time of testing, details of the history of the samples are unknown. However, in this case it was unlikely that milling was the source of the blocked lysine, as the samples were most likely milled on a small scale mill, rather than an industrial mill that can result in high local temperatures.

The mOPA and ninhydrin methods gave similar results of average blocked lysine contents for most sites and cultivars. On average, for all flour samples, 12% reactive lysine loss was measured by both the ninhydrin method and the mOPA method (standard error of values for 36 flours: 1% for each method). This is quite high, considering these flour samples had undergone no processing other than milling, and reiterates the importance of testing for lysine content in feed ingredients if the nutritional quality of feed is important. For the samples from Balfour, the blocked lysine content shown in Figure 2.2-i equates to, on average, 25% of the total lysine in the flour samples grown at this site (standard error of 4 samples: 2% for each method).

Good correlation between the ninhydrin method and the mOPA method for assessing lysine contents and blocked lysine contents of barley flours has been shown. This indicates that the mOPA method is a valid technique for assessing the relative lysine contents of proteins with low water solubility. Furthermore, the mOPA method is sensitive to early Maillard reaction damage. As it is also suitable for feed with free lysine added, the mOPA method was used in chapter 4 to analyse reactive lysine loss during feed pelleting.

The mOPA method is also technically simple, involves no heating or hydrolysis step, and allows for a high throughput of samples. This makes it ideal for testing large numbers of samples quickly and accurately. It is particularly useful in situations where sample homogeneity is an issue, and where samples are being compared for reaction before and after processing. For industrial applications, the method would need to be adapted, as

suggested by Bertrand-Harb *et al*, to stabilise the chromophore, and remove the need for precise timing during the assay.[109]

However, this study does emphasize the difficulty of finding absolute values for lysine contents in complex systems, which will always be complicated by issues such as the reactivity of α-amino groups and free amino acids in the system. To give absolute values of lysine content in a system, results have to be adjusted for the amino groups present. In addition, the inherent variation observed from using different testing methods highlights the requirement to perform a growth trial with chickens to gain definitive results. Nevertheless, without any adjustments, these tests are very useful for comparative measurement of lysine contents for the same system, which has undergone different treatments, as was undertaken in this thesis.

Subsequently, others from this laboratory have utilitised this modified OPA method, further showing its utility in a variety of systems. Rasiah has correlated the mOPA method with the TNBS method for the testing of wheat proteins.[110] Wheat proteins were extracted from frozen croissants in four fractions – the albumin/globulins, gliadins, SDS-soluble glutenins and SDS insoluble glutenins. Overall, the correlation between the two methods very good, with correlation from 0.70-0.89.[110] This would indicate that the mOPA method is also suitable for wheat proteins. Gerrard *et al.* and Brown also utilised the mOPA method for following the loss of reactive lysine in wheat proteins which had undergone Maillard reaction.[111,112]

2.5.4 The increase in amino group concentration during incubation under Maillard reaction conditions

To limit potential errors from protein concentration measurements, resulting from the low response of the Bradford assay to RNase A (section 2.4.2), a series of incubations were performed without subsequent dialysis. Interestingly, the results of these incubations showed increases in amino group concentration as incubation progressed. In this study, a trend of increasing apparent lysine availability was seen as the incubation progressed and the lysine contents of incubated samples were significantly less than the incubated controls, indicating protein break-down. This led to an analysis of the role of dialysis and

pH in model systems involving the incubation of xylose or glucose with RNase A in aqueous solution, at 50°C. Glucose and xylose were chosen for this study as they are pure carbohydrates, and allowed techniques to be established prior to use with feed carbohydrates, which may give rise to many side reactions due to their complex nature.

Comparison of dialysed and non-dialysed RNase A incubations and controls

A study investigating the effect of dialysis on lysine assays, using xylose or glucose as the model reducing compound, was undertaken. These experiments clearly showed that increasing incubation time led to increased amino group analysis readings, if dialysis was not performed. Figure 2.5-k illustrates, that in undialysed samples, the amino group content increases steadily over the period of incubation, whereas the dialysed samples have a small but steady loss of amino groups.

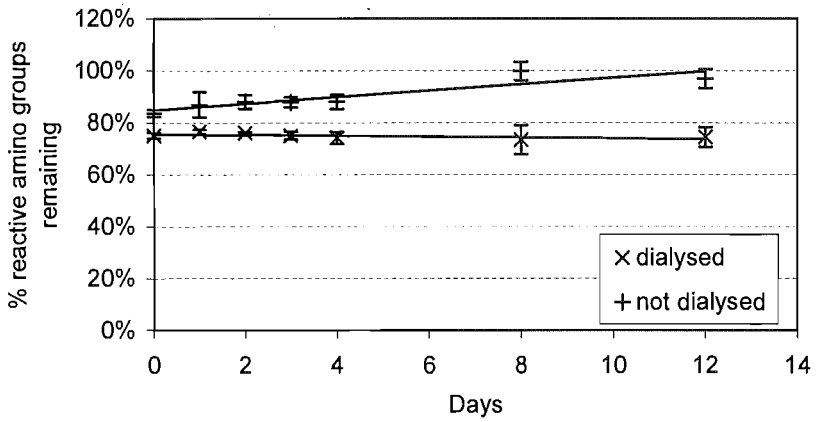


Figure 2.5-k: Percentage of amino groups remaining after incubation of RNase A with carbohydrate at 50°C, assayed by the OPA method (results of four incubations averaged for each point), showing the significant difference between dialysed and undialysed samples. Error bars represent standard error about the average of four measurements.

This increase is most probably due to the break-down of protein during the incubation of the samples, leading to an increase in N-terminal protein ends, which led to larger amino group assay results. While the presence of lower molecular weight protein fragments in

some control samples (Cf, Ci) was obvious by SDS-PAGE (Figure 2.5-c), the occurrence of these fragments was generally more apparent in SDS-PAGE gels only after incubation with a carbohydrate, as illustrated by Figure 2.5-l. This was consistent with the increased fragmentation of RNase A after incubation as postulated from amino group assay results (Figure 2.5-k).

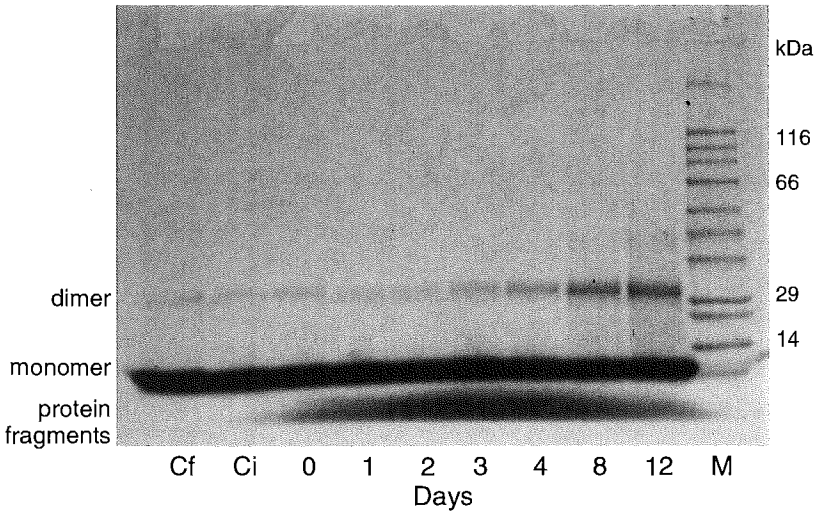


Figure 2.5-l: Typical SDS-PAGE gel of 1.8 mM RNase A incubated in aqueous solution with 137 mM glucose at 50°C, pH4, for 0-12 days, showing the presence of small molecular weight protein fragments after incubation. Cf: Frozen control; Ci: incubated control (RNase A only); M: Sigmamarker (wide-range).

These N-terminal amino groups are also available to undergo reaction with carbonyl groups *via* the Maillard reaction. Therefore, the analysis of lysine count data may be compromised by the occurrence of these two competing reactions. Sirnilar results have been seen in other reactions in our laboratory, where Maillard reacted wheat albumins and globulins had lower lysine counts than unreacted protein, but the lysine count increased with reaction time.[111]

Dialysis will reduce the amount of small protein fragments in the sample, but fragments may have undergone crosslinking to the monomer such that they are hidden by the monomer band on the SDS-PAGE gel, and no longer removed by dialysis. This would be expected to happen to a greater degree at longer incubation times, adding to the difficulty in analysis of lysine count data.

When the controls for all the incubations were analysed, the data were consistent with protein break-down during incubation, as the measured amino group concentration for incubated controls of RNase A for all samples were consistently higher than frozen controls (Figure 2.5-m).

At a pH of 4, the difference between reactive amino group contents of the frozen and incubated controls was much less than for samples at pH 7. Increasing the pH to 7 appeared to exacerbate the protein break-down of the incubated control solutions markedly, with a difference between the incubated and frozen control reactive amino group concentration of 41% for undialysed samples, and 76% for dialysed samples (Figure 2.5-m). Presumably this increase is due to a lower stability of RNase A at pH 7, above which irreversible unfolding can occur,[113] causing the protein to fragment more rapidly during the incubation period. No effect of this change in pH was observed for the frozen controls.

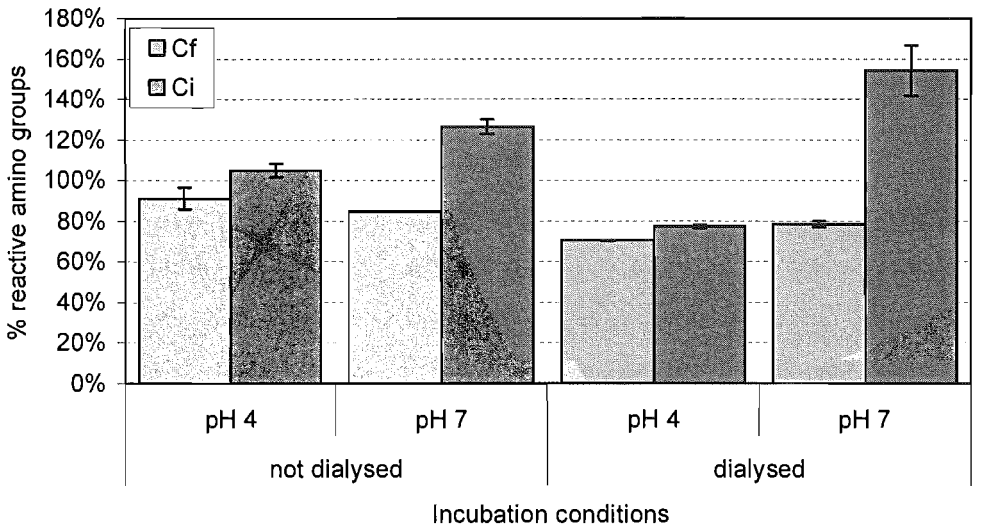
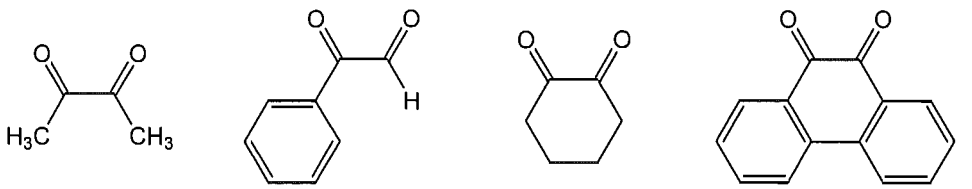


Figure 2.5-m: Average amino group content of control samples (1.8 mM RNase A) which were frozen after preparation (Cf), or incubated at 50°C for 12 days (Ci). The effect of dialysing controls and pH on amino group content of the samples are compared. All results relative to the theoretical amino group content of 1.8 mM RNase A. Error bars represent the standard error of two replicates.

2.6 ARGININE TESTING

Arginine is an essential amino acid for poultry,[114] and is also known to take part in the Maillard reaction, through its guanidine group.[115-122] Hence, the loss of arginine is pertinent when investigating the impact of the Maillard reaction on nutritional quality. The literature in this area is much smaller than that for lysine testing however. One of the earliest qualitative tests for arginine was based on an observation by Sakaguchi in 1925, where certain guanidine derivatives were reported to give a colour change on reaction with α-napthol at high pH.[123,124] The popularity of this method, with various modifications that have occurred over time, was such that Smith and MacQuarrie noted, in 1978, that the Sakaguchi method appeared to be the most commonly used selective quantitative method for arginine determination.[125] In general, around 10 mg of protein is required for arginine determination *via* this method, which can make the cost of testing certain proteins excessive.[125]

Today, the more common method for determining arginine concentration appears to be through automated amino acid analysis, but this consumes reasonably large amounts of protein and involves acid hydrolysis, which introduces limitations as discussed for lysine assays.[125] In many cases, modification of arginine in proteins is for structure-function studies, and for this purpose a range of dicarbonyls are used, such as butanedione, phenylglyoxal and cyclohexanedione (Figure 2.6-a).[124] The later two reagents are made radioactive for quantitation, but it is difficult to make butanedione radioactive, and hence quantitate.[124]



2,3-butanedione phenylglyoxal cyclohexanedione 9,10-phenanthrenequinone

Figure 2.6-a: Structures of various reagents used to modify arginine residues.

A more convenient method for testing arginine content, which is 1000 times more sensitive than the Sakaguchi method, was developed by Smith and MacQuarrie in 1978.[125] This

method uses 9,10-phenanthrenequinone (Figure 2.6-a), a diketo compound that forms a stable fluorescent compound upon reaction with the guanidine group of arginine.[125] Unlike the Sakaguchi method, the protein does not need to be hydrolysed prior to analysis,[125] making it a more suitable method for Maillard reacted residues. In addition, the method has been tested with RNase A, and has been shown to have good sensitivity and linearity with this protein.[125]

The 9,10-phenanthrenequinone method of Smith and MacQuarrie[125] was modified for use in a microtitre plate reader for this research. This simply involved scaling the sample size down to a volume more suitable for the microtitre scale. Calibration curves with RNase A showed this to still be a valid method on the reduced scale (Figure-2.6-b), although error was increased slightly, from 1.4% to 3.4%. However, as it allowed a large number of samples to be assessed quickly, compared to the more laborious standard method, this reduced method was used in further analysis of arginine content in RNase A following incubation with cyclotene, as discussed in chapter 3.

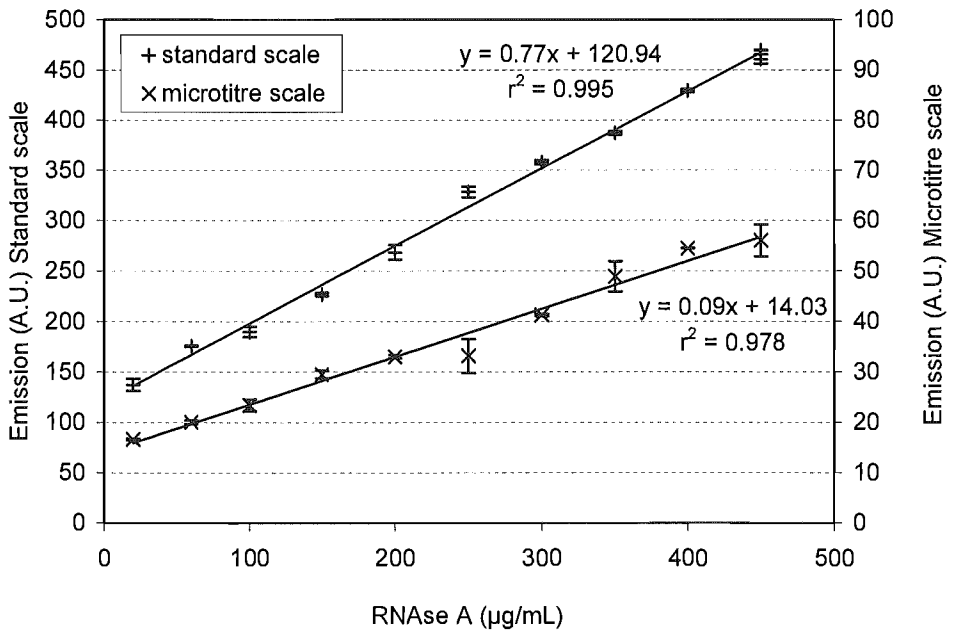


Figure-2.6-b: Comparison of calibration curve for arginine testing of RNase A on a standard (9 mL) and microtitre (200 µL) scale, at medium voltage. Measurements performed in duplicate with error expressed as standard error of duplicate measurements.

2.7 CONCLUSIONS

Lysine is an essential amino acid for both poultry and humans, and can be as a result of processing or storage. In order to determine the degree of this loss, a method for testing the lysine content of various food and feed systems is required. Unfortunately lysine testing is complex, due to the acid lability of non-digestible lysine complexes and the general loss of protein digestibility that can occur during the Maillard reaction. Many *in vivo* and *in vitro* methods have been developed to test for lysine in a variety of situations, none of which are ideal under all circumstances. *In vivo* techniques tend to be expensive and time consuming, whereas *in vitro* techniques may not be as applicable to *in vivo* situations. The choice of lysine testing method depends on the situation being analysed.

For this thesis, RNase A was chosen as the model protein for analysis (chapter 3). The Bradford method for testing protein concentration was modified for use with RNase A, which has a low response with the Bradford solution. Barley flour was chosen as a model for chicken feed for studies described in chapter 4.

It was decided to follow the progression of Maillard reaction in model systems *via* pH. However, as small sample volumes led to decreased pH as a result of carbon dioxide solubility, this method was not used. However, in a system using amino acids or inexpensive protein in reaction with carbohydrates over a short time scale, this method could be useful.

The OPA method for lysine analysis was chosen as the most suitable for model systems containing RNase A. The ninhydrin method was chosen for systems containing barley flour, but was found not to be suitable for systems without high homogeneity, or with high concentrations of lysine. Therefore the OPA method was successfully adapted for use with proteins extracted from barley flours. This modified method showed good concurrence with values gained from the ninhydrin method.

Difficulties were encountered in the study of RNase A. It was found, that despite being a stable protein, it still underwent fragmentation on incubation and at increased pH. These problems are likely to be exacerbated in cereal systems. Dialysis of samples did not reduce the effect of this on lysine analysis.

Arginine is an essential amino acid for chickens, and may also undergo destruction *via* the Maillard reaction on processing or storage. To analyse arginine loss for this thesis, the phenanthrenequinone method was modified for use on a microtitre scale.

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INVESTIGATING THE MAILLARD REACTION IN MODEL SYSTEMS

CHAPTER 3

3.1 BACKGROUND

In chapter 1, the importance of the Maillard reaction in feed systems was discussed. Chapter 2 investigated and established methods for studying the progress of the Maillard reaction in model systems. Prior to studying applied systems in more depth (chapters 4 and 5), the progression of the Maillard reaction in relatively uncomplicated single protein systems was examined.

A considerable degree of Maillard reaction research has involved the use of model systems to investigate specific aspects of this group of reactions. In general, this research employed a simple model system, containing either an amino acid or dipeptide, and a carbonyl containing molecule.[1] Most of the current knowledge of the Maillard reaction has been gathered from the study of these systems, due to the inherent complexity of the *in vivo* and *ex vivo* environments in which Maillard chemistry occurs.[2] However, the evidence to date suggests that important differences in chemistry occur between amino acids and proteins,[3] and thus a protein system was employed in these model systems.

As described in chapter 1, an understanding of how crosslinking occurs in proteins, *via* the Maillard reaction, and the nutritional consequences of this, are very relevant. Model protein systems were studied as part of this thesis to understand more about the basic mechanisms of the Maillard reaction, building on current knowledge, and what this means for reactive lysine loss, protein crosslinking and the involvement of other amino acid residues. In feed processing, high temperatures are used for a short period of time. In order to monitor the chemistry in model systems, lower temperatures were utilised for a longer time period. Specifically examined was the occurrence of crosslinking in proteins during the Maillard reaction, and the relation of this to loss of reactive amino groups.

3.2 THE REACTION OF RNASE A IN MODEL SYSTEMS

With the methodology for following the Maillard reaction selected and optimised, as outlined in chapter 2, a variety of carbohydrates were investigated for reactivity in a model protein system. The pure carbohydrates glucose, xylose and cyclotene (Figure 3.2-a) were chosen to give a clear understanding of the progression of the Maillard reaction in the model system, as they have been extensively studied in model systems with respect to the Maillard reaction.[2,4-11]

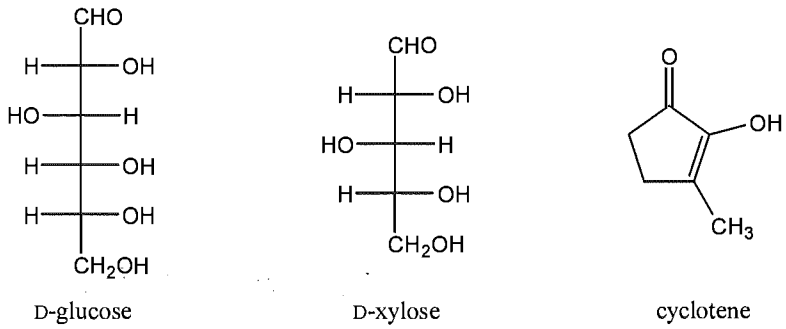

Figure 3.2-a: The carbohydrates glucose and xylose (drawn as the Fischer projection)[12] and cyclotene (depicted in the enol form).[2]

Cyclotene (2-hydroxy-3-methyl-cyclopent-2-enone) (Figure 3.2-a) is a volatile flavour compound, common in food, which is known to undergo Maillard chemistry and lead to protein crosslinking.[2] It is both a natural product, found in foods such as coffee beans, and a synthetic food additive.[2] Many carbonyl compounds known to take part in the Maillard reaction have unstable structures prone to redox chemistry, leading to difficulties in their study.[2] Cyclotene, however, has a simple, stable structure that is not prone to redox chemistry, making it an ideal molecule for study under Maillard reaction conditions.[2]

Cyclotene had previously been shown in our lab to be an ideal carbohydrate to incubate with RNase A, due to clear crosslinking profiles on SDS-PAGE and good mass spectrometry results.[2] Research published by Gerrard *et al.* has shown, by electrospray mass spectrometry techniques, that initially one molecule of cyclotene reacts with lysine within RNase A and subsequent dehydration follows to form a Schiff base intermediate.[2] It

has been proposed that this Schiff base is the key intermediate in the protein crosslink formation with cyclotene.[2] Mass spectrometry results detailed in the same study showed that four of the ten lysine residues in RNase may be involved in the reaction.[2]

The feed-type carbohydrates studied included sucrose, malt extract, dextrin I, starch and molasses. These are described further in section 3.6. They were studied to assess how a variety of carbohydrates, and carbohydrate mixtures, would react with RNase A, and hence how the Maillard reaction may progress during feed processing.

3.3 THE INITIAL REACTION OF RNASE A WITH CYCLOTENE, XYLOSE OR GLUCOSE

Incubations were performed with RNase A and cyclotene, xylose or glucose, in order to determine the role of carbohydrate type and incubation temperature on protein crosslinking.

The initial drop in lysine content

The most consistent result found across incubations was an immediate loss in amino group content, for most carbohydrates tested. Despite immediate freezing, on mixing the carbohydrate with RNase A, an immediate loss of amino groups in RNase A was found (Figure 3.3-a). This initial loss in amino group content was probably due to the immediate formation of early Maillard reaction products, such as the Schiff base or Amadori product. While the Schiff base is formed before the Amadori product, the formation of the Schiff base has been shown to be the rate determining step in some Maillard reaction systems.[13] This is pertinent, as it indicates that heating above room temperature is not required to induce the Maillard reaction in these systems.

Similar results were seen by Gerrard *et al.*, after incubating RNase A with the more reactive carbonyls glutaraldehyde and formaldehyde, which showed an immediate loss in amino groups of almost 100% and 20% respectively.[14] This corresponded with immediate

crosslinks formation for both incubations,[14] which was not seen for any incubations with the less reactive carbohydrates used in these incubations.

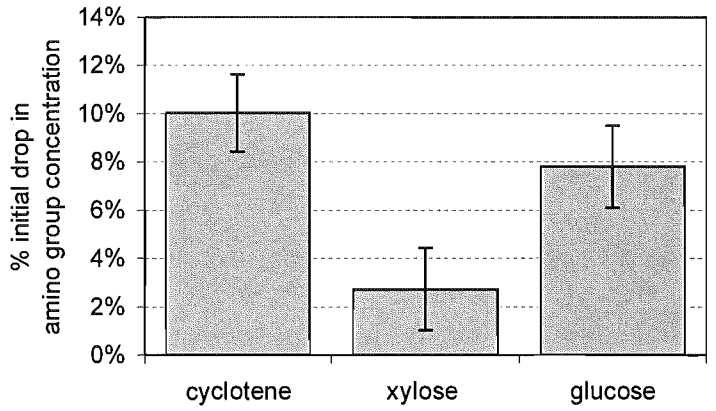

Figure 3.3-a: The average percentage loss of amino groups from RNase A after addition of a carbohydrate, where samples were frozen immediately after preparation. The drop is shown relative to frozen control for each experiment. Error bars represent standard error of replicate incubations (3-11 replications).

The difference in amino group concentration drop after immediate reaction of RNase A with cyclotene, xylose or glucose may have been as a result of cyclotene readily forming a Schiff base,[2] whereas glucose, present mainly in its cyclic form, reacted less rapidly.[15] Xylose is known be present in its acyclic form in very low concentrations,[16] possibly explaining the low initial reaction.

3.4 Long Term Reaction of RNase A with Cyclotene

RNase A was incubated with cyclotene for a period of up to a month. The aim of this study was to fully explore the relationship between reactive amino group loss and crosslinking of RNase A. The crosslinking profile of this incubation is shown in Figure 3.4-a, and the amino group concentrations throughout the incubation are shown in Figure 3.4-b.

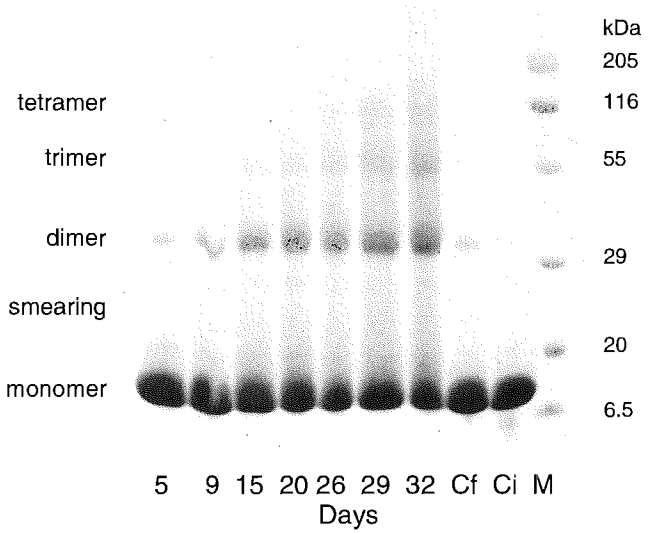

Figure 3.4-a: A typical SDS-PAGE gel of 1.8 mM RNase A incubated in aqueous solution with 130 mM cyclotene at 37°C, pH 4, for 5-32 days (undialysed). Cf: Frozen control; Ci: incubated control (RNase A only); M: Sigmamarker (wide-range).

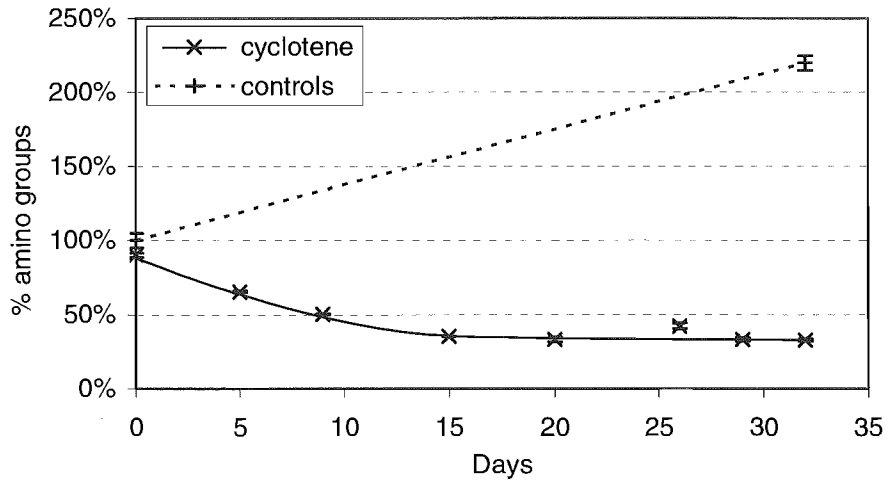

Figure 3.4-b: Loss of reactive amino groups in RNase A after incubation with cyclotene at 37°C, pH 4 (dialysed). All data are shown relative to the frozen control. Error bars represents standard error of triplicate measurements.

The SDS-PAGE gel shown in Figure 3.4-a, clearly displays the smearing effect that was often visualised after Maillard reaction had occurred in model systems. This smearing effect has been previously observed when RNase A was incubated with dehydroascorbic

acid,[3] and has been attributed to the formation of protein fragments which are subsequently crosslinked, resulting in products of many different sizes.[3] If correct, the lysine count would reflect a balance between a gain in extra free amine groups at the N-terminus of the new protein fragments and loss of amino residues due to Maillard reaction. The data shown for this experiment probably reflected these competing processes.

In Figure 3.4-b, after an initial drop, reactive amino group loss can be seen at 5 days, before any crosslinking is evident on inspection of the SDS-PAGE gel (Figure 3.4-a). Furthermore, by the fifteenth day of incubation, the rate of amino group loss has levelled out, while crosslinking continued beyond this point. Interestingly, the lysine availability reduced to a minimum of 40%, which was also the minimum lysine availability seen by Gerrard *et al.* after incubation of RNase A with the more reactive formaldehyde or glyceraldehyde.[14] This may have implications for the reactivity of surface lysine residues in RNase A.

The comparison between crosslinking rate and amino group loss gave some insight into the crosslinking mechanism. There are a number of possible mechanisms by which the Maillard reaction can proceed to crosslinking in proteins (Figure 3.4-c). Crosslinking *via* the Maillard reaction could occur by a lysine residue reacting with a reducing sugar to form a Schiff base, and subsequently reacting with another lysine residue to form the crosslink, (Figure 3.4-c-(i)). Alternatively, other residues such as arginine, could participate in the crosslink (Figure 3.4-c-(ii)). This has been observed in previous experiments with glucose and other sugars.[17-20] Another possibility involves the reaction of two derivatised lysine residues reacting to form a crosslink (Figure 3.4-c-(iii)). The above result that amino group loss was minimal (Figure 3.4-b), while crosslinking continued (Figure 3.4-a), indicates that option (i) could only occurring if protein fragmentation, producing reactive N-terminal amino groups, was occurring at the same rate. The occurrence of protein fragmentation during this incubation was supported by control data, if the assumption is made that the presence of cyclotene was not altering the rate of protein breakdown. However, given the literature evidence for the involvement of arginine residues in the carbohydrate induced crosslinking reactions of proteins,[17,21,22] an independent measure of arginine concentration was undertaken.

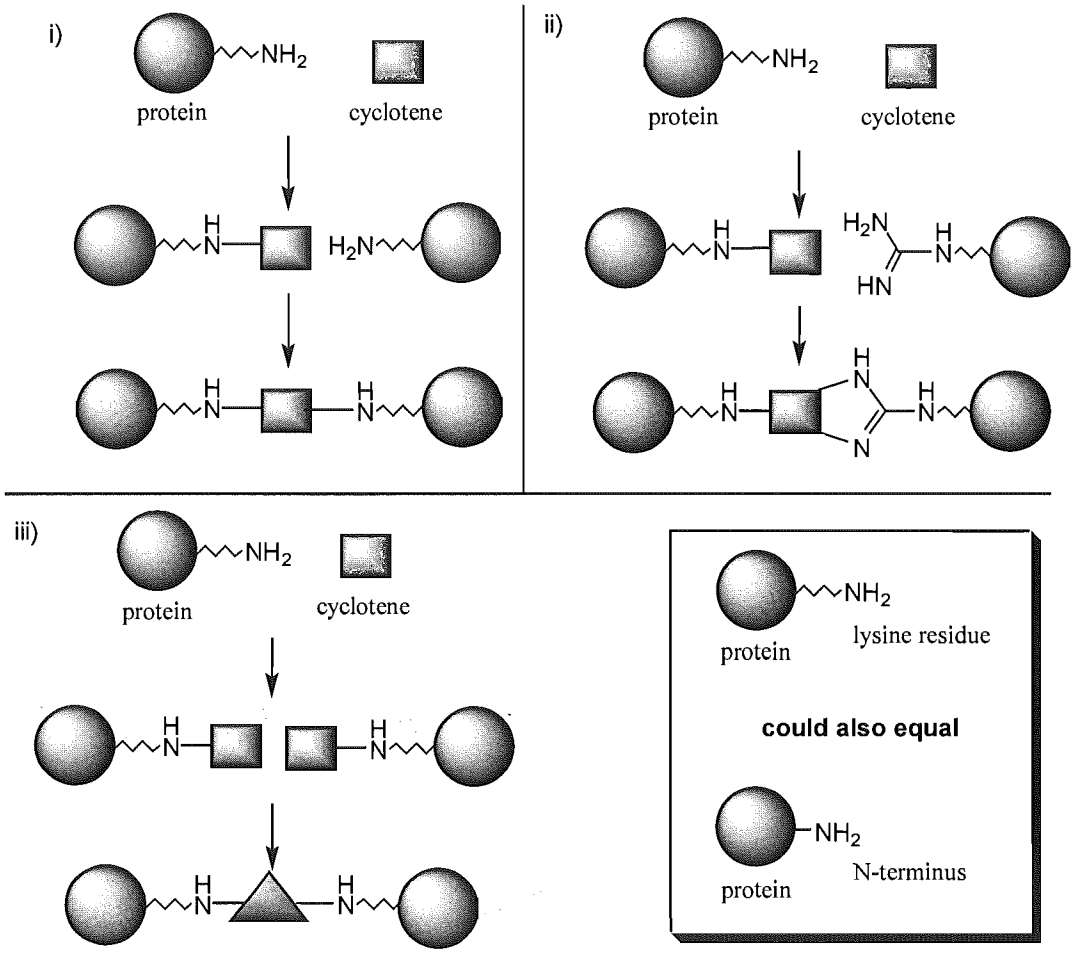

Figure 3.4-c: Stylised representation of the possible reaction pathways for RNase A crosslinking to occur when incubated with cyclotene. Pathways described in text.

The role of arginine in the RNase A/cyclotene reaction

Arginine has been shown to react with reducing sugars and lysine residues to form crosslinks in a number of protein systems.[17,23-25] Pentosidine, formed on reaction of glucose with lysine and arginine, is probably the most common of these crosslinks reported in the literature, although it has been reported to be only a minor crosslink product in food systems[26] and in the body.[24] In the study performed here with RNase A and cyclotene, at 37°C, no major loss of arginine was seen during incubations (Figure 3.4-d), indicating it was not involved significantly in crosslinking reactions. This was consistent

with the findings reported by Gerrard *et al.*, who determined that Schiff base formation occurs *via* the lysine residue but not the arginine residue.[2]

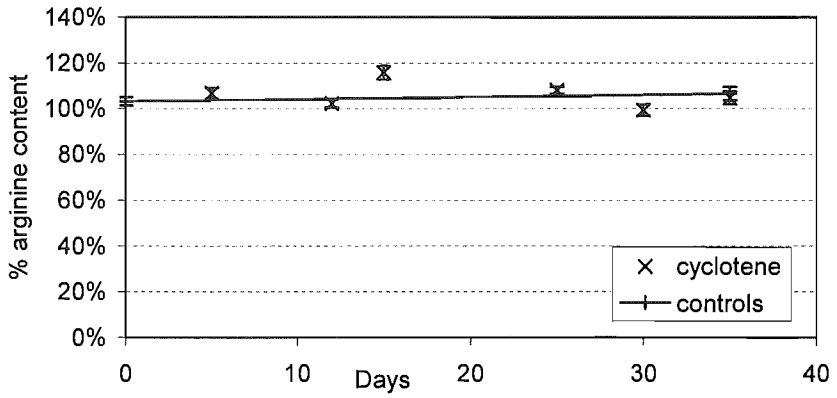

Figure 3.4-d: Arginine remaining in RNase A after incubation with cyclotene at 37°C, pH 7, 5-35 days (dialysed). All data are shown relative to the frozen control. Error bars represent standard error of 5 measurements.

As no loss of arginine was seen during reaction, this residue was not taking part in crosslinking reactions to any large extent. This discounts the possible role of arginine, as postulated in Figure 3.4-c (ii). Therefore, it was likely that derivitised lysine residues were reacting together to form the crosslinked protein, or that crosslinking was occurring at the same rate as protein fragmentation formed reactive N-terminal amino groups. Therefore, no further analysis was performed to investigate arginine modification in model systems.

3.5 THE REACTION OF RNASE A WITH CYCLOTENE, XYLOSE OR GLUCOSE

The 37°C incubation of RNase A with cyclotene was repeated over a shorter, 0-8 day timescale, and parallel incubations were performed at 50°C and 70°C to gauge effect of temperature on the Maillard reaction. In conjunction, incubations of RNase A with xylose or glucose were also performed at 37°C, 50°C and 70°C, for comparison of crosslinking rate and amino group change with varying carbohydrate type.

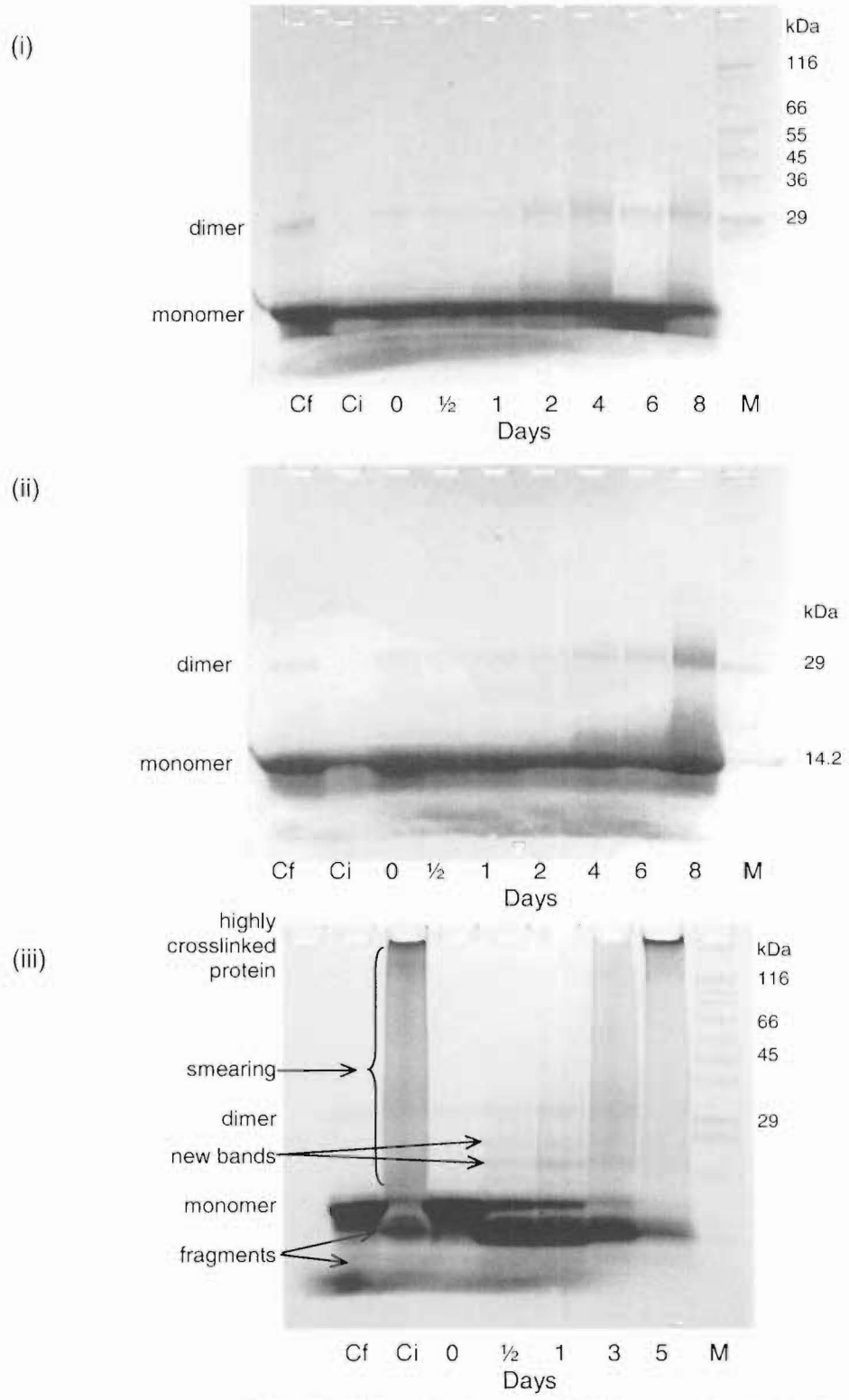

Figure 3.5-a: Typical SDS-PAGE gels of 1.8 mM RNase A incubated in aqueous solution with 130 mM (i) cyclotene, 50°C; (ii) xylose, 50°C; (iii) glucose, 70°C; pH 7, (undialysed). Cf: frozen control; Ci: incubated control (RNase A only); M: Sigmamarker (wide-range).

Incubations of RNase A at 70°C, in aqueous solution, were performed to more closely mimic the temperatures encountered during feed processing. Determining how RNase A reacts at increased temperature and in the presence of various carbohydrates may give insight into the processes occurring during the processing of chicken feed, and whether these reactions can be controlled to minimise the reduction of nutritionally available lysine.

None of the incubations of RNase A with carbohydrate showed any evidence of crosslinking by SDS-PAGE gel after 8 days at 37°C, and similarly, no crosslinking was apparent after incubation with glucose for 8 days at 50°C. However, RNase A reacted with cyclotene or xylose to show evidence of crosslinking after 2 or 4 days of incubation at 50°C respectively (Figure 3.5-a(i) and (ii)).

The appearance of RNase A incubated at 70°C, with or without carbohydrate, on SDS-PAGE gels was very different to those seen at 50°C. After incubation with glucose for 12 hours most of the monomeric RNase A band had broken apart to produce two distinct fragments both at about 8 kDa, as well as smaller fragments visible on the SDS-PAGE gel (Figure 3.5-a(iii)). The two fragments were observed in the incubated control, indicating that the fragmentation was a result of temperature. In addition, at 12 hours, protein bands were visible between the monomer and dimer bands, presumably as a result of crosslinking reactions between the protein fragments. This fragmentation was also seen after incubation with xylose or cyclotene. This indicates that RNase A fragmented in a consistent manner, to produce distinct fragments which crosslinked with the monomer, to give two well defined bands, at approximately 20 and 23 kDa (Figure 3.5-a(iii)). After 5 days of incubation with glucose, most of the RNase A was visible as highly crosslinked protein that could not enter the gel (Figure 3.5-a(iii)). This was more pronounced after incubation with either xylose or cyclotene, where after 3 days incubation at 70°C, little protein was able to enter the gel due to the large degree of crosslinking. At this temperature the incubated control also showed significant smearing, and the presence of protein aggregates at the top of the gel.

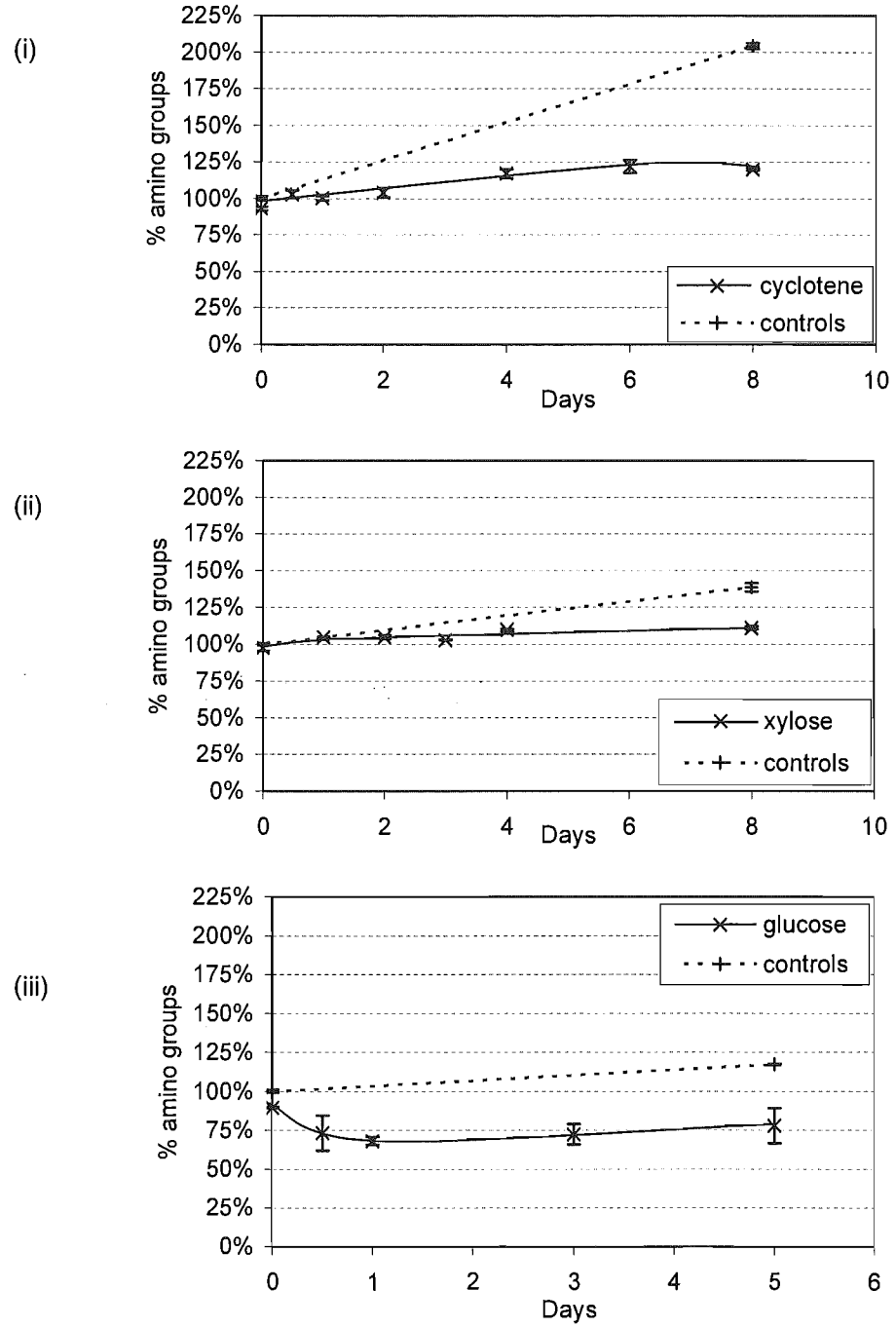

Figure 3.5-b: Typical amino group contents of RNase A after incubation with cyclotene, xylose or glucose at (i) 37°C; (ii) 50°C; (iii) 70°C; pH 7 (undialysed). All data are shown relative to the frozen control. Error bars represents standard error of 3 measurements.

The amino group counts profile for incubation between RNase A and cyclotene at 37°C (Figure 3.5-b(i)) appeared quite different for to that seen in Figure 3.4-b, where the amino group content reduced to approximately 50% after incubation for nine days. In the 8 day incubation, after a zero time decrease in amino group content, as seen previously (section 3.3), the amino group content for this experiment increased slowly over the incubation period. Similar results were seen for reaction between RNase A and xylose or glucose at 37°C. This increase was most likely due to protein fragmentation, as discussed in chapter 2.

The two incubations (Figure 3.4-b and Figure 3.5-b(i)) of RNase A with cyclotene at 37°C were consistent in the overall amino group loss seen, however. In the second incubation (Figure 3.5-b(i)), amino group content for the 8 day sample was 84% lower than the incubated control for the same time period. This was similar to the reduction in amino group content seen after 9 days in the previous experiment, indicating both sets of data were valid. However, this variation highlights the difficulty of changing batches of RNase A between experiments, suggesting that the former batch had been less prone to fragmentation, as discussed in chapter 2.

In Figure 3.5-c, the processes underlying the amino group counts are estimated graphically. While, in the calculation of these lines, the large assumption was made that the processes occurring during incubation of protein occurred at a linear rate, they do explain the amino group profiles seen after incubation of RNase A with carbohydrate at 37°C (Figure 3.5-b (i)) relatively well. The 'fragmentation' line in Figure 3.5-c was calculated on the assumption of a linear increase in the amino group content of the incubated control from Figure 3.5-b (i), due to the formation of N-terminal ends. Over the 8 days of the experiment, an increase of 104% was seen, which equated to 13% per day. The "overall" line in Figure 3.5-c, is the best fit line for the cyclotene incubation data, and indicates an average increase of 3% per day. It was assumed that the difference between these two lines (*i.e.* 10% per day) was due to Maillard reaction processes blocking the reactive amino groups. The 'Maillard' line in Figure 3.5-c shows this postulated reaction process, as it would be observed if no fragmentation was occurring. This shows how a high fragmentation rate can mask the Maillard reaction effect on amino group contents of incubated samples, even though lysine residues may still be rendered nutritionally unavailable.

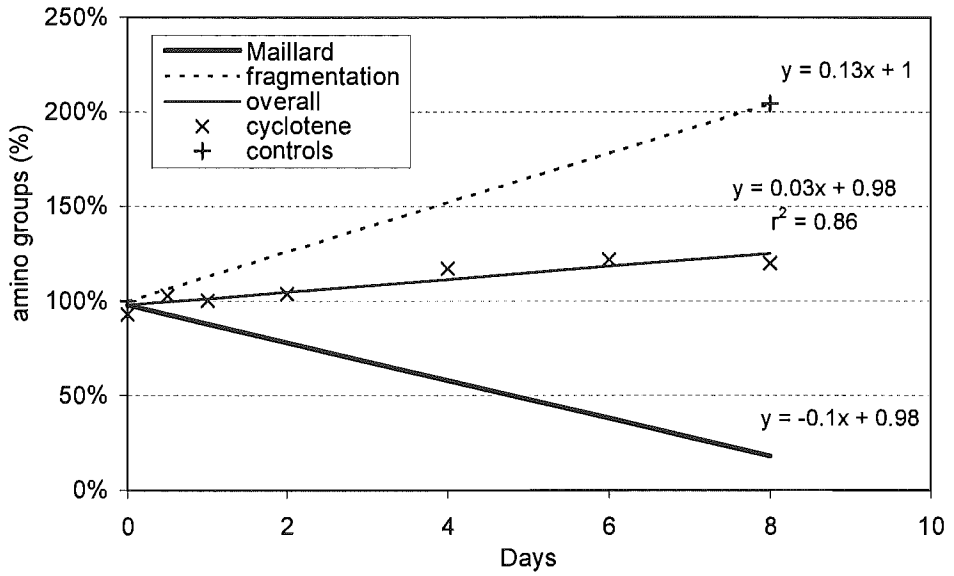

Figure 3.5-c: Theoretical processes underlying the overall change in the amino group content visible after incubation of RNase A. Details explained in text. Standard error (cyclotene 1%; controls 2%) not shown, as obscured by data points.

After reaction of RNase A with carbohydrate at 50°C, the amino group profile increased for 1-2 days then levelled out (Figure 3.5-b(i)). It was therefore obvious that the assumption of linearity did not hold over the entire incubation period, and more complex processes were occurring. The control, incubated at 50°C, had fewer reactive amino groups than the equivalent control incubated at 37°C. As the rate of protein fragmentation would be expected to increase with temperature, this was probably due to protein aggregation and precipitation occurring, and consequential steric hindrance during the amino group assay. This was more clearly seen in the 70°C incubated control (Figure 3.5-b(iii)).

The amino group profile of RNase A, after incubation with cyclotene or glucose at 70°C, decreased for the first 12-24 hours and then slowly increased over the remainder of the incubation period (Figure 3.5-b(iii)). During the same time period, little change in the amino group content of RNase A incubated with xylose occurred. The amino group content of the incubated control increased to 117% over 5 days at 70°C.

For RNase A incubated with or without carbohydrate, the change in amino group content seems minimal, given the crosslinking profile over the same time (Figure 3.5-b(iii)). This indicated that the reduction in reactive amino groups *via* the Maillard reaction and steric hindrance was occurring at approximately the same rate as protein fragmentation was forming reactive N-terminal groups. For the incubated control, the small change in amino group content was probably due to the aggregation of incubated control proteins, as seen by SDS-PAGE, and consequential steric hindrance during the assay.

3.5.1 Summary of the reaction of RNase A with cyclotene, xylose or glucose

Immediately on mixing RNase A with the carbohydrates cyclotene, xylose or glucose at room temperature, a reduction in amino group content of 3-10% occurred. This indicated that immediate reaction between the protein and carbohydrate had occurred, such that the modified amino groups were no longer reactive in the OPA assay.

From the SDS-PAGE data, increasing temperature from 37°C to 70°C dramatically increased the rate of crosslinking, Similarly, from SDS-PAGE data, the carbohydrate reactivity with RNase A appeared to be cyclotene≈xylose>glucose at 50°C and 70°C. The amino group profiles of these experiments clearly showed the conflicting processes which occur during incubation of RNase A with carbohydrates. It was apparent, that with these carbohydrates the process of fragmentation produces amino groups at approximately the same rate that Maillard reaction processes modify them, under many of the conditions used in these experiments. The concurrent aggregation processes that occur with increased temperature in incubated controls made it more difficult to prove that the Maillard reaction was responsible for the reduction in amino groups in samples incubated at higher temperatures.

The results in this section emphasised the importance of monitoring macromolecular changes to the protein, simply achieved by SDS-PAGE, in conjunction with lysine count data, in order to assist interpretation of the data.

3.6 THE REACTION OF RNASE A WITH FEED-TYPE CARBOHYDRATES

The feed-type carbohydrates used for this incubation series with RNase A were sucrose, starch, dextrin, malt extract and molasses. Sucrose is a disaccharide, obtained mainly from sugar beet and sugar cane. It is a non-reducing sugar, and hence should not react with proteins under Maillard reaction conditions. Starch is a large polysaccharide, containing few reducing ends, and hence was expected to be unreactive.[27] Dextrin is a starch hydrolysis product, containing 1→6 linkages.[27] Approximately 10-20% of dextrin consists of reducing matter,[28] which may react under Maillard conditions. Malt extract is an extract of malted barley, which consists mainly of the reducing disaccharide maltose (~60%),[29] along with various oligosaccharides, inorganic salts and protein.[30] Molasses is a by-product of sucrose refinement, containing approximately 50% sugar, in a mixture of mainly mono and disaccharides.[31,32] It is included in poultry feeds to stimulate appetite, reduce feed dustiness, and improve pellet durability.[32-34]

The initial drop in lysine content with feed-type carbohydrates

In section 3.3, an immediate reduction in the reactive amino group content of RNase A occurred on mixing with cyclotene, xylose or glucose (Figure 3.3-a). With the carbohydrates molasses and malt extract, a similar effect was seen (Figure 3.6-a). Starch and sucrose produced no significant change in the amino group content of RNase A, as would be predicted from their lack of reducing moieties. Dextrin also produced no significant reduction in the amino group content of RNase A immediately after mixing, indicative of the presence of relatively few reducing moieties compared with the more reactive carbohydrates. The relatively large standard errors were probably due to the complex composition and inherent impurities in feed-type carbohydrates, as large standard errors were not seen for the pure carbohydrates or sucrose, the most pure of the feed-type carbohydrates.

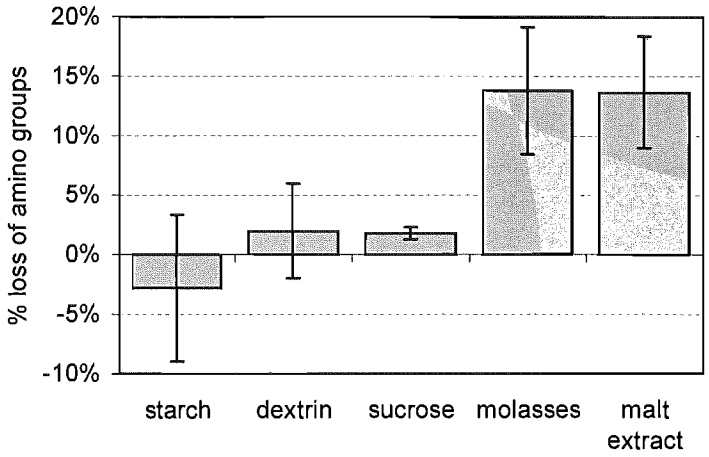

Figure 3.6-a: The average percentage loss of amino groups from RNase A after addition of a variety of carbohydrates, where samples were frozen immediately after preparation. Values are shown relative to the frozen control. Error bars represent standard error of 3 replicate incubations.

For analysis purposes, the incubations of RNase A with feed-type carbohydrates were separated into two groups. The first group was comprised of starch and sucrose, which were not expected to react significantly with RNase A, due to their lack of reducing ends. The second group consisted of malt extract, dextrin and molasses, all of which contain reducing sugars, and were expected to react with RNase A.

3.7 THE REACTION OF RNASE A WITH STARCH OR SUCROSE

After incubation with sucrose (Figure 3.7-a(i)) or starch for 8 days at 37°C, no changes in RNase A were visible by SDS-PAGE. At 50°C, a gradual reduction in the density of the monomer band of RNase A was visible (Figure 3.7-a(ii)), with a concomitant increase in protein fragmentation obvious on day 8 of the incubation. The 8 day incubated sample was very similar to the incubated control, indicating this was the background change in RNase A during control incubation at 50°C. The smearing at the edges of the bands visible at zero time and day 1 starch incubations was due to the starch, which had not fully dissolved and was visible in the sample, preventing the protein entering the gel evenly.

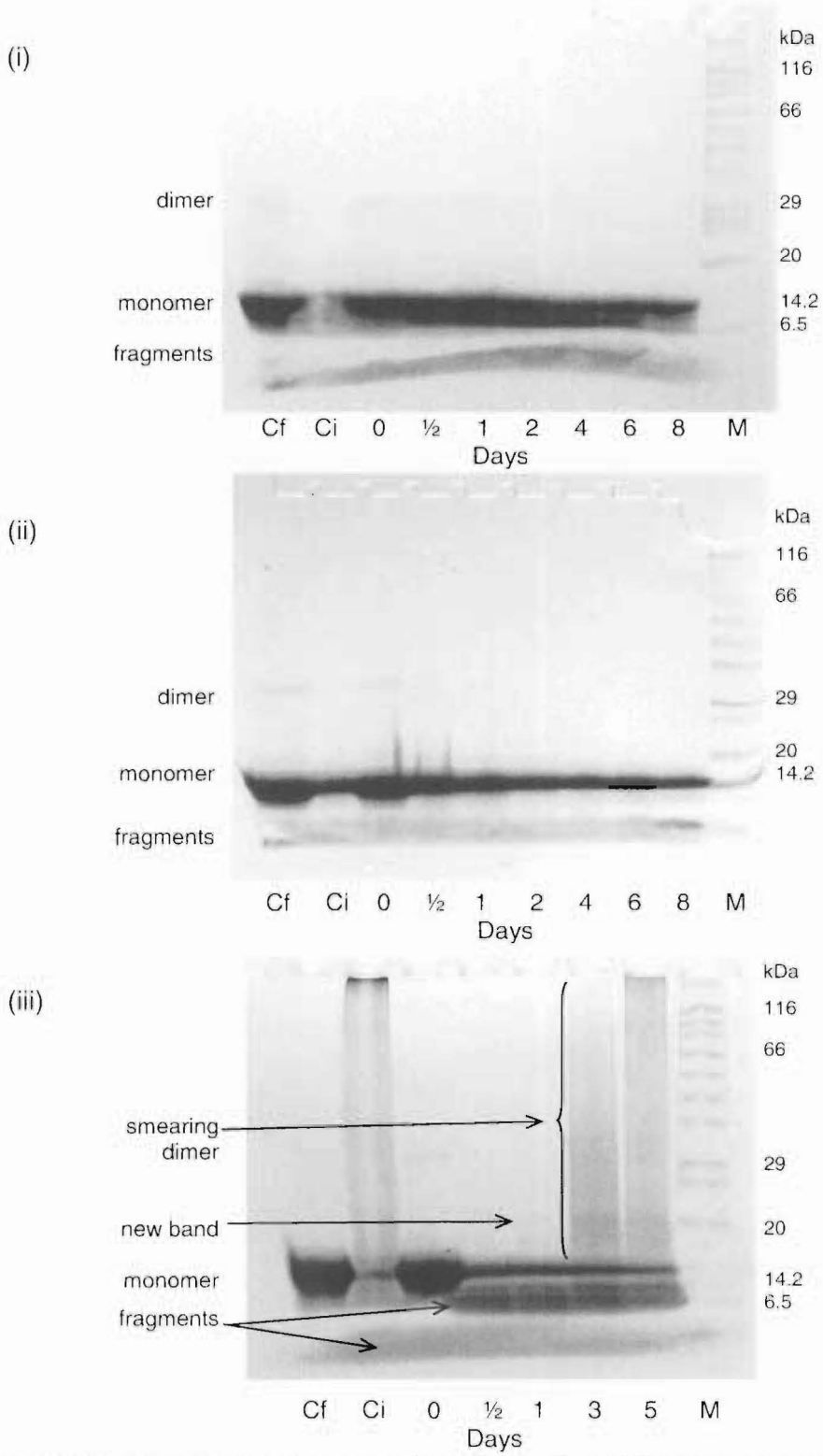

Figure 3.7-a: Typical SDS-PAGE gels of 1.8 mM (25 mg/mL) RNase A incubated in aqueous solution with (i) 130 mM sucrose at 37°C; (ii) 25 mg/mL starch at 50°C; (iii) 130 mM sucrose at 70°C; pH 7, (undialysed). Cf: Frozen control; Ci: incubated control (RNase A only); M: Sigmamarker (wide-range).

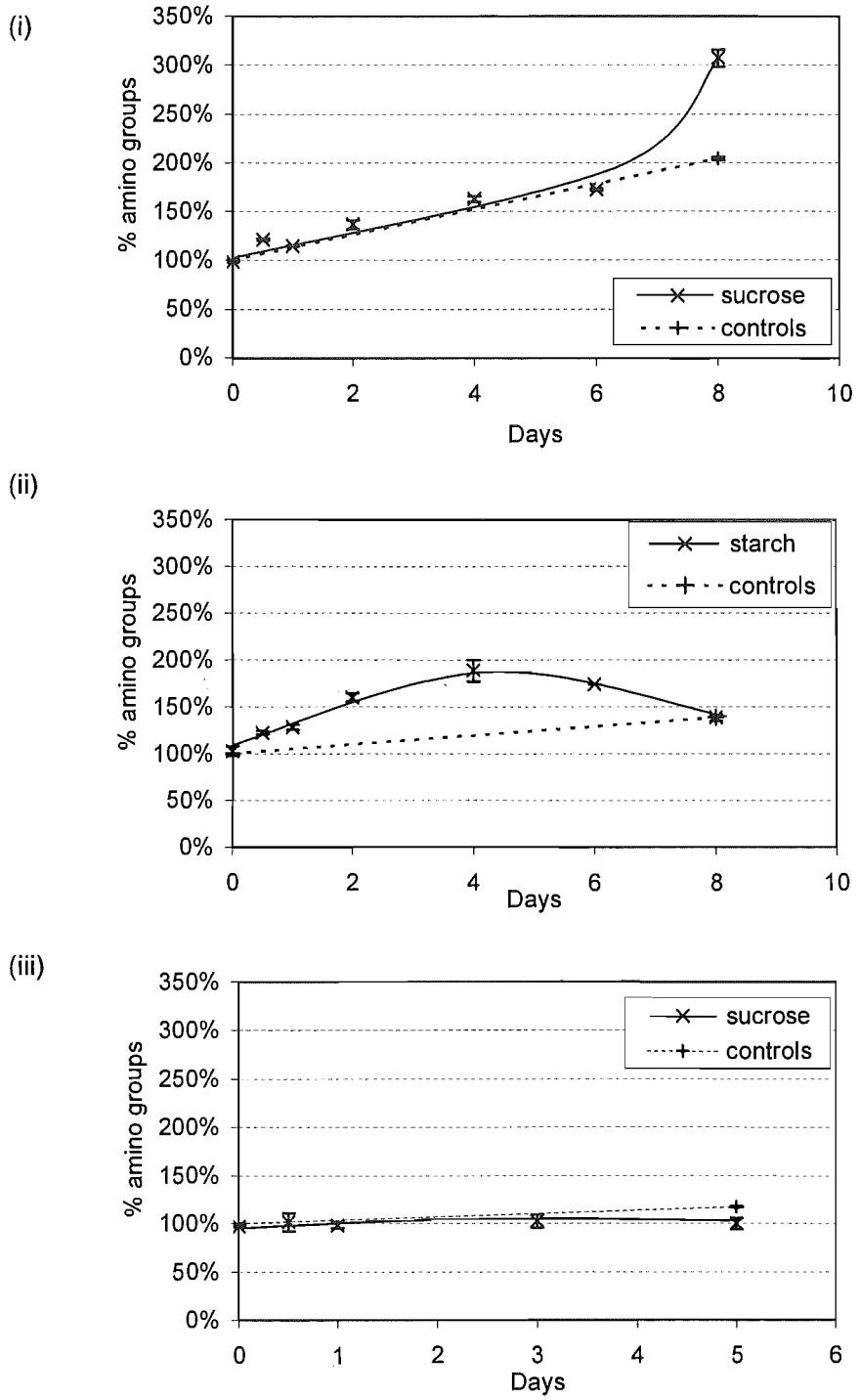

Figure 3.7-b: Typical amino group contents of RNase A after incubation with starch or sucrose at (i) 37°C; (ii) 50°C; (iii) 70°C; pH 7 (undialysed). All data are shown relative to the frozen control. Error bars represent standard error of triplicate measurements.

Reaction of RNase A with starch or sucrose at 70°C was very similar to that of RNase A with glucose, as visualised by SDS-PAGE (Figure 3.7-b(iii)). Smearing of the protein increased over the 5 day incubation, but monomer was still visible at 5 days. The presence of the band at 20 kDa after 12 hours indicates some crosslinking reaction was occurring, however the presence of monomer after 5 days indicates that this was to a much lower degree than seen for glucose (Figure 3.5-a(iii)).

The amino group profile of RNase A after incubation at 37°C with either carbohydrate, showed a slow increase in the number of amino groups over the first 6 days, followed by a sharp increase for the 8 day incubation (Figure 3.7-b(i)). This indicates increased fragmentation in these 8 day samples. While the amino group profiles seen after incubation of RNase A with starch or sucrose at 50°C were different to that at 37°C, they were again comparable with each other (Figure 3.7-b(ii)). The decrease in reactive amino groups by day 8 may be indicative of hydrolysis of starch and sucrose occurring over this time period, producing reducing moieties which are reacting with the protein fragments.

The amino group count for RNase A incubated at 70°C with starch and sucrose changed little over 5 days (Figure 3.7-b(iii)), indicative of the opposing processes of protein break-down and protein aggregation, both obvious on the SDS-PAGE gel (Figure 3.7-a(iii)).

3.8 The reaction of RNase A with molasses, malt extract or dextrin

Incubation of RNase A with malt extract at 37°C or 50°C, or with dextrin at 37°C, showed slight protein fragmentation and no evidence of crosslinking, producing SDS-PAGE gels similar to Figure 3.7-a(i). However, molasses incubations at 37°C and 50°C (Figure 3.8-a(i) and (ii)), and dextrin incubations at 50°C, produced large amounts of protein fragments. The monomer band gradually decreased during the 50°C incubation period, consistent with fragmentation occurring.

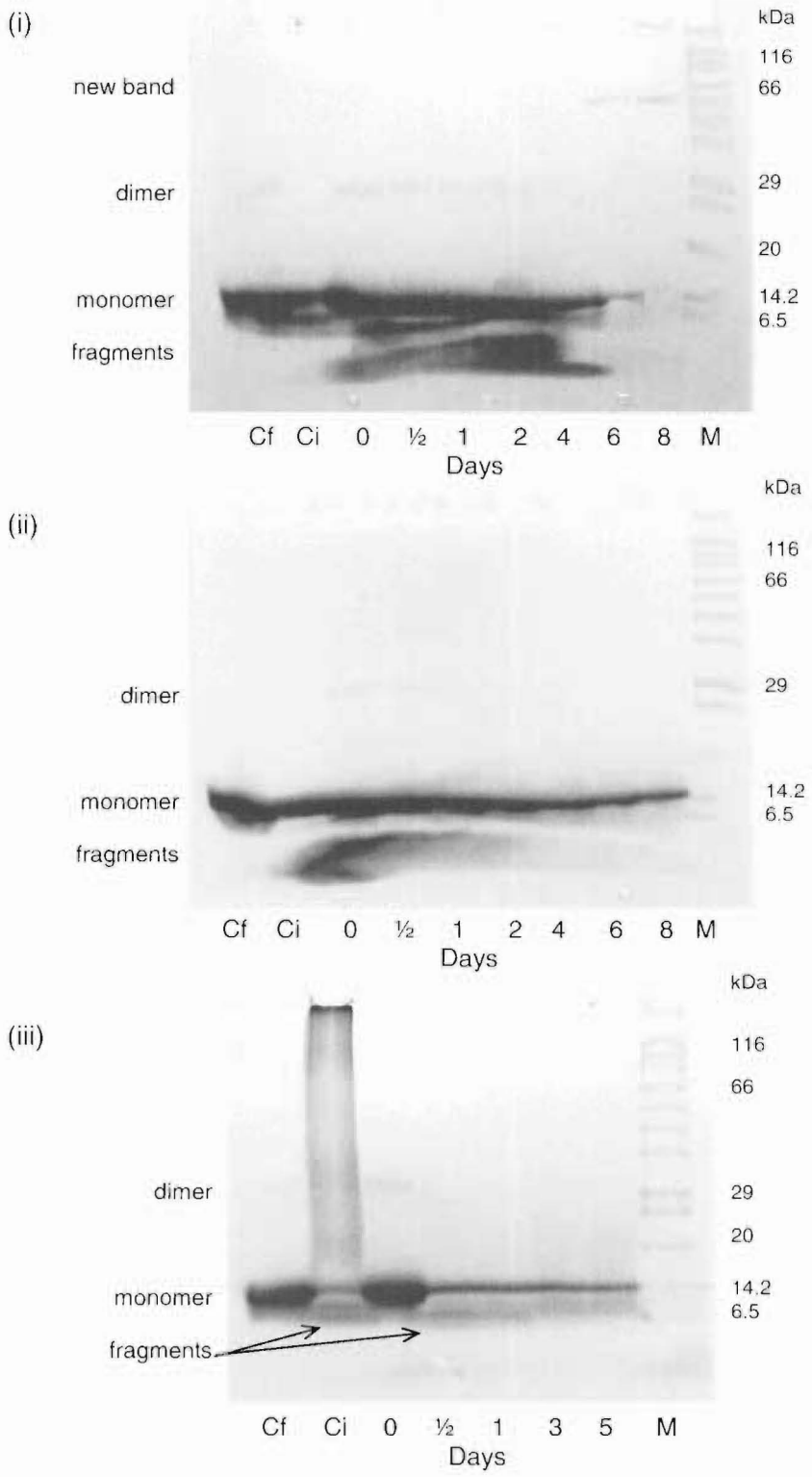

Figure 3.8-a: Typical SDS-PAGE gels of 1.8 mM (25 mg/mL) RNase A incubated in aqueous solution with 25 mg/mL (i) molasses at 37°C; (ii) molasses at 50°C; (iii) molasses at 70°C; pH 7; (undialysed). Cf: Frozen control; Ci: incubated control (RNase A only); M: Sigmamarker (wide-range).

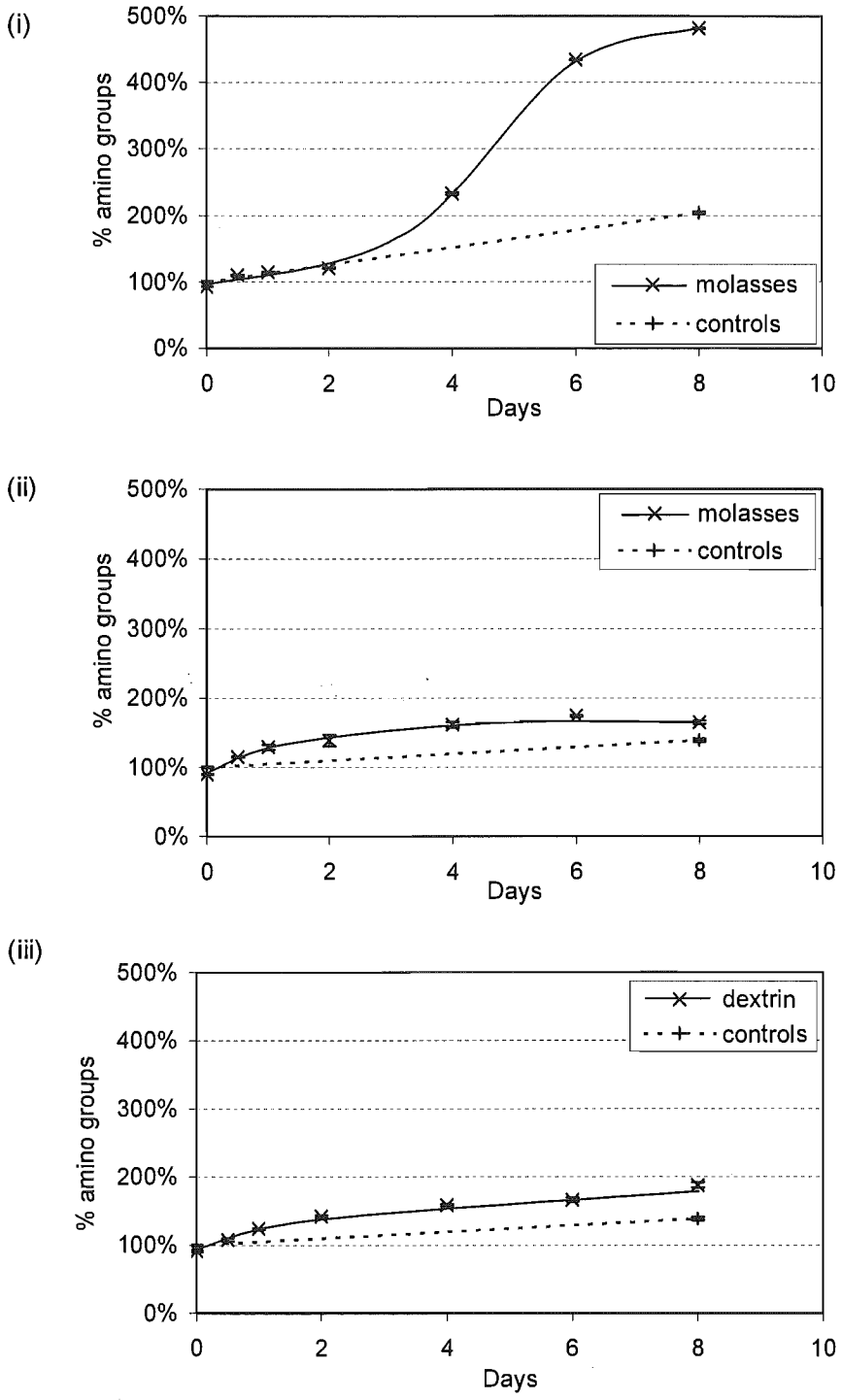

Figure 3.8-b: Typical amino group contents of RNase A after incubation with malt extract, molasses or dextrin at (i) 37°; (ii) 50°C; (iii)70°C; pH 7 (undialysed). All data are shown relative to the frozen control. Error bars represent standard error of triplicate measurements.

After 6-8 days incubation with molasses at 37°C, a greatly reduced quantity of protein was observed on the gel and a new band was visible at about 55 kDa (Figure 3.8-a(i)). As no highly crosslinked protein was obvious at the top of the gel, it was most likely that most of the protein had fragmented, possibly as a result of free radical reactions catalysed by trace metals present in molasses, such as iron (~130 ppm) and copper (~14 ppm).[35-38] Protein fragmentation has previously been shown to occur after glycation of protein in the presence of metals.[39-41] This phenomenon was replicated in repeat incubations of RNase A with molasses. The large increase in amino group content (Figure 3.8-b) observed on days 6-8 confirms that the protein had fragmented significantly at these incubation periods, to produce many reactive N-terminal amino groups. This large increase in amino group content was not seen after incubation of RNase A with either malt extract or dextrin, which had profiles similar to that seen for cyclotene or glucose.

The reaction of RNase A with dextrin at 70°C was comparable to that with sucrose at 70°C, as seen by SDS-PAGE (Figure 3.7-a(iii)), whereas reaction with malt extract produced a greater amount of highly crosslinked protein, similar to that seen after incubation with glucose (Figure 3.5-a(iii)). In contrast, on incubation with molasses, RNase A appeared to fragment into well defined bands to a lesser degree, with monomer predominant after all incubation times (Figure 3.8-a(iii)).

The amino group profiles of RNase A incubations at 37°C with malt extract or dextrin were similar to that observed in Figure 3.5-b(i). At 50°C and 70°C the amino group profiles for all incubations of RNase A with molasses, dextrin or malt extract followed a similar pattern, with only slight variations (Figure 3.8-b(ii) and (iii)). Over the first 1-2 days, the amino group content increased relatively steeply, and then increased slightly over the remaining period of incubation.

3.8.1 Summary of the reaction of RNase A with starch, sucrose, dextrin, malt extract or molasses

As seen with the pure carbohydrates (section 3.3), immediate reduction in the reactive amino group content of RNase A occurred on mixing with the carbohydrates sources of

molasses and malt extract. This was not seen after mixing with starch, sucrose or dextrin, which is consistent with their lack of reactive reducing moieties.

From the SDS-PAGE data, starch and sucrose did not appear to react significantly at any incubation temperature. This was expected, due to their lack of reducing moieties. No crosslinking was evident, by SDS-PAGE, after incubation of RNase A at 50°C with any of the feed-type carbohydrates. Therefore none of the feed-type carbohydrates were as reactive as xylose or cyclotene, under these conditions. The feed-type carbohydrates did increase protein fragmentation in many incubations, however. Molasses produced the most varied results of the feed-type carbohydrates, causing large amounts of protein fragmentation to occur in some incubations.

It is apparent from amino group data and SDS-PAGE that incubation of RNase A with complex mixtures of carbohydrates, such as malt extract or molasses, can result in more complex reactions occurring, especially an increased rate of protein fragmentation. This highlights the difficulty of *in vitro* testing of lysine content in systems where fragmentation is occurring, and confirms the importance of monitoring the macromolecular effects along with amino group counts.

3.9 CONCLUSIONS

These experiments were conducted to determine the effect of carbohydrate type, and incubation temperature, on the behaviour of RNase A under Maillard reaction conditions. This was measured by examining crosslinking and fragmentation patterns on SDS-PAGE gels, and the concurrent amino group profiles of these reactions.

As detailed in section 3.5, the conflicting processes of protein fragmentation, producing reactive N-terminal amino groups, and modification of amino group *via* the Maillard reaction, produce the amino group profiles seen. At 37°C the amino acid profiles of RNase A incubated with xylose, cyclotene, glucose, malt extract or dextrin were relatively uncomplicated, suggesting near linear rates of both processes. However, incubation with molasses, starch or sucrose, or increasing the temperature above 37°C, led to more complex profiles as a result of increased rates of protein fragmentation, as well as

aggregation occurring in the control samples. This highlights the problem of testing systems for amino group content when fragmentation is occurring concurrently, and while these experiments can give an indication of what is occurring, growth trials are necessary if nutritional availability of lysine is to be accurately determined.

Overall, SDS-PAGE gels gave a clearer indication of the rate of reaction of RNase A under various incubation conditions. In general, cyclotene and xylose appeared to be the most reactive of all of the carbohydrates tested, showing the most crosslinking at all temperatures employed, as determined by SDS-PAGE. Arginine did not appear to be an important moiety in the crosslinking reaction between cyclotene and RNase A.

The reaction of sucrose and starch with RNase A appeared to be insignificant under the conditions employed in this study. Feed-type carbohydrates gave results, both by SDS-PAGE and amino group profiles, which were more complex than for the pure carbohydrates, indicative of the wider variety of reactive moieties present in these impure systems. Results indicated that starch and sucrose are more suited to adding to feed pellets than the more reactive molasses or malt extract.

The high degree of protein aggregation, seen in both incubated controls and samples at 70°C, indicates that nutritional quality of processed feed may decrease with or without the occurrence of Maillard reaction.

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LYSINE LOSS DURING THE PELLETING PROCEDURE

CHAPTER 4

4.1 BACKGROUND

In chapter 1, the importance of the poultry industry, the need to minimise feed cost, and the possibility of the Maillard reaction occurring during processing were discussed. In this chapter, the potential for loss of bioavailable lysine to occur during feed processing in the New Zealand poultry industry is directly addressed. First, the dietary requirements of poultry will be covered in greater detail, along with how feed is formulated to meet these requirements, and subsequently processed. Studies that have investigated the potential for the Maillard reaction to occur in feed will then be detailed.

The projected global production for poultry meat is close to 100 million metric tons per year for 2005.[1] In New Zealand, 70 million broiler (meat) chickens are raised each year.[2] There are many issues to be considered in the poultry industry, in part due to the consumer focus on food safety and animal welfare issues, while still requiring a consistent, affordable, quality product.[3] There is consumer and political pressure, especially in European countries, for chickens that have been raised without antibiotics and on a diet free from genetically modified ingredients.[3] This increases the demand for improved management practices, new biotechnology and optimised diets.[3] Intensifying the requirement for an optimised diet is the factor that the cost of producing feed is by far the greatest expenditure involved in the production of any intensively reared animal.[4] For example, feed production makes up 60-70% of total broiler production costs.[5]

In light of this information, it is obvious that any loss of nutritional quality during the processing of feed is of great interest to the feed manufacturers. If the Maillard reaction is occurring during the processing of feed, this could mean damage, and hence nutritional loss, of the free lysine and other free amino acids added to the meal. In addition, the

occurrence of the Maillard reaction could lead to the formation of antinutritional compounds,[6] which could interfere with gastric enzymes or be toxic to the bird,[7] as well as mutagenic and antimutagenic compounds.[8] Previous research into this area is discussed further in section 4.1.3.

4.1.1 The dietary requirements of poultry

The dietary requirements of poultry are not uniform for all birds. The major factors that affect nutrient requirements are outlined in Table 4.1-a.

	Factors affecting nutrient requirements
Environmental	• Environment temperature/humidity • Type of housing (*e.g.* cage, wire or litter floor) • Presence of environmental stress factors • Restricted feeding – either an effect due to limitations of feeder space or imposed as a management tool • Destruction of nutrients by ultraviolet light • Destruction of nutrients in feed or drinking water by nitrites, sulphites or other chemicals
Bird/digestion	• Genetic make-up • General bird health – disease presence • Presence of intestinal parasites • Influence of hormones in the bird and from animal by-product ingredients • Intestinal pH • Presence of beneficial/detrimental intestinal bacteria • Damage to absorptive cells, or lack of digestible fat or bile, leading to decreased absorption • Competition for absorption due to nutrient imbalances or lack of factors needed for active nutrient absorption
Feed	• Energy content of feed • Nutrient availability • Presence of oxidising fats, especially in the presence of catalysing minerals and lack of antioxidants • Presence of fungal toxins in feedstuffs • Nutrients unavailable due to colloids in ingredients • Nutrients unavailable due to adverse interactions with other nutrients in the intestinal tract • Positive/negative effects of enzymes found in ingredients • Presence of anti-metabolites in certain feedstuffs

Table 4.1-a: Factors that can influence bird nutrient requirements. Adapted from Leeson and Summers.[9]

Nutrient needs not only vary between poultry species, but can vary between strains.[10] In chickens, for example, the breed; the purpose of the bird – whether it is for egg production (layer) or meat production (broiler); and the stage of life (chick to adult, moulting, laying *etc.*) all have an affect on what the dietary requirements will be.[10]

The formulation of chicken feed

Poultry diets are formulated to meet the needs of the particular animal, and usually depend on what ingredients can be easily and economically sourced in the locality of the industry. For example, in the North Island of New Zealand, broiler diets are based on maize produced in the North Island, and are similarly based on wheat or barley in the South Island, both supplemented by grains imported from Australia and/or Canada.[11] On average, diets contain just under seventy percent grain.[12] In the largest poultry producing nations – China, Brazil and the USA – corn and soybean meal are produced locally, and hence tend to be the basis of poultry diets.[12]

Other ingredients are added to the diet to meet various other requirements of the birds. Fat, in the form of animal tallow or vegetable oils, is usually included.[10] Different minerals and vitamins are generally required, depending on specific deficiencies within the diet.[10] Enzymes are often added to hydrolyse diets, or to assist in the metabolism of antinutritive elements in a diet.[13] For example, β-glucanases are added to barley-based diets to improve the digestibility of the non-starch polysaccharides known as β-glucans, and hence limit their anti-nutritive effects.[13]

To meet animals' amino acid requirements, supplementation with feedstuffs containing high quality protein is required, as mentioned in chapter 1.[14] In addition to soybean meal, high quality protein ingredients such as blood meals, fish meals, meat meals and feather meals are often included. Synthetically derived individual essential free amino acids are also added to the diet, as this is more economical than increasing the levels of high quality protein, such as soybean.[15] The most common amino acid to be included is lysine, as it is limiting in most poultry diets,[15] and hence relatively large amounts are often added to maximise growth. Depending on the particular diet, other essential amino acids such as methionine, threonine, tryptophan and arginine may also be added.[15]

As free amino acids contain a primary amino group, they are susceptible to Maillard chemistry. Reducing sugars are present in low levels in grains and maize,[16] and in high levels in soybean meal and molasses.[17,18] There is also potential for lipids in the diet to increase Maillard reaction rates, as lipids from soybean oil have been shown to increase the rate of Maillard reaction under laboratory conditions.[19]

If free amino acids are undergoing reaction, it is probable that they will no longer be nutritionally available. Hence, additional free amino acids would have to be added to the diet to account for this loss during processing. Lysine is particularly susceptible to reaction, as it contains two primary amino groups. This has been shown experimentally by Baxter, who found a loss of 28% in lysine content for a glucose/amino acid mixture after sterilization at 128°C for 225 seconds, compared with a loss of 8-14% for other amino acids, including methionine, threonine and tryptophan.[20] The susceptibility of methionine, tryptophan and threonine to undergo the Maillard reaction has also been established.[7,21,22]

Measuring broiler performance

The *in vivo* assay section of chapter 2 detailed numerous methods for examining the bioavailability of lysine in poultry diets. Most of the methods discussed can also be used for determining the dietary requirement of various nutrients. Diets are formulated based on the performance in growth trials, and more specific measurement methods, of the bird in question.

In general, growth trials are used to determine the growth rate of birds on various diets, as well as other variables such as feed efficiency and mortality. A key determinate of feed value is the feed conversion ratio (FCR) (Equation 1), which measures feed efficiency. The FCR is of obvious importance, as the majority of the cost of bird production is derived from the cost of feed,[23] and the FCR describes how well this feed is converted into animal weight, and hence economic return.

$$\text{FCR} = \frac{\text{feed intake over time period (g)}}{\text{weight gain over time period (g)}} \qquad \text{Equation 1}$$

There are naturally considerations other than feed efficiency. Obviously, bird mortality is an issue, which can potentially occur as a side-effect of high growth rates. In addition, consumers' preference for lean chicken meat must be taken into account,[24] moving the focus from bird mass to protein production.[24]

4.1.2 Feed processing

The intensive livestock industry is one in which margins are small and competition fierce. Hence, any factor that can be altered to increase animal performance may increase profits markedly. It has been well established that pelleting feed has positive effects on the FCR of up to 12%,[5] and this has been attributed to improved palatability, enhanced diet homogeneity, lower microbial content and improved digestibility, amongst other factors.[4] In addition, pelleted feed has the advantage of better flow properties, important for easy movement in conveyance equipment, and a higher density, resulting in larger quantities capable of being stored and transported.[25]

The benefits of pelleting, in dollar terms, must obviously outweigh the increased cost of the pelleting process for it to be economical, and this cost/benefit analysis must also be applied to any change in feed components or processing methodology. In general, the objectives of pelleting include optimisation of nutritional quality (energy, protein quality), hygienic quality (bacterial count), and physical qualities (hardness and durability).[25] The importance of increasing the quality of pellets is being increasingly recognised by industry members, due to the resulting benefits.[24]

There are three stages of feed processing which have the greatest potential to result in lysine damage through the Maillard reaction: grinding, conditioning and pelleting (Figure 4.1-a). All three involve increase in temperature and pressure, and during conditioning steam is also added to the process. It is well accepted that high temperature and intermediate moisture conditions contribute to an increase in the rate of Maillard reaction.[26] It has also been shown that pressure accelerates the formation of the Amadori product and pentosidine.[27,28]

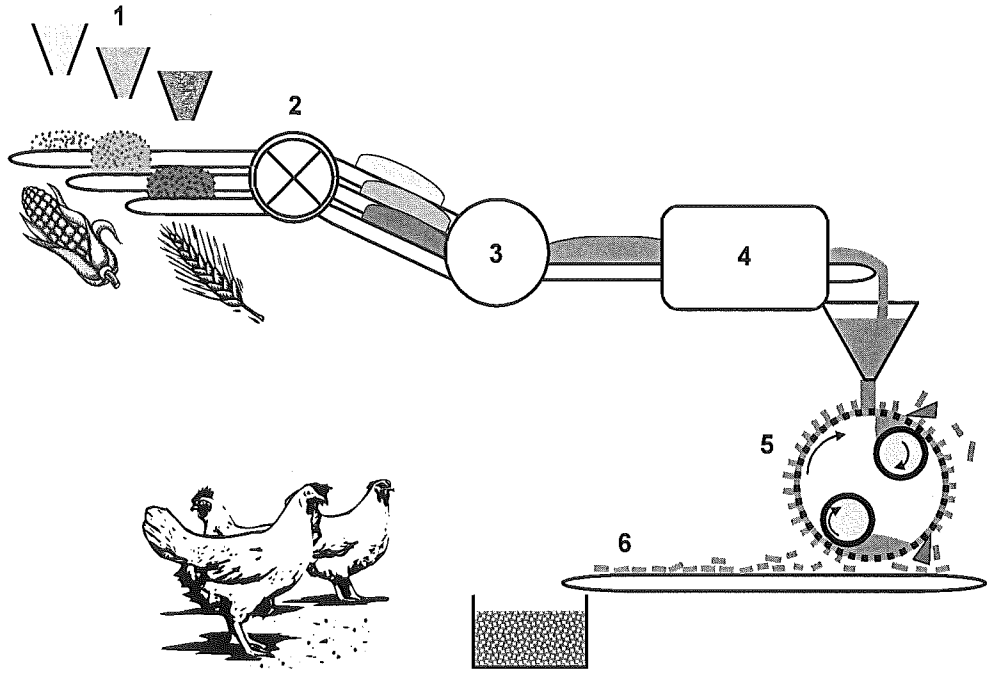

Figure 4.1-a: Stylised representation of the pelleting process. 1) Ingredients added in correct ratios; 2) Ingredients ground (these first two steps may be performed in reverse order); 3) Ingredients mixed into meal; 4) Meal conditioned; 5) Meal pelleted; 6) Pellets cooled and dried then sifted and transported to poultry farms.

Grinding

Feed ingredients are ground for a variety of reasons. Size reduction is important for ingredients that are normally too large to be included in broiler diets, such as maize kernels or faba beans.[29] The process of grinding also increases access to the inner parts of the grain for digestion by enzymes, as well as improving functional qualities such as ease of blending, and mash formation.[29]

The most common method of dry milling is *via* the hammer mill, which uses force to shatter the feed material. Energy consumption is high, with outputs of up to 200 kW/tonne of wheat depending on grain type and required particle size. This causes a rise in temperature that is partially offset by the evaporation of water in the feed material. It may

be at this stage that most damage occurs to grain protein, but this is difficult to assess, as unmilled samples cannot be assayed. However, in future work, amino acid analysis could be used, in conjunction with lysine analysis methods designed to assess Maillard damage, to give some idea of the lysine loss in this phase, as described for flour samples in chapter 2.

Conditioning

Conditioning is a term used in animal feed manufacturing to describe a process whereby feed meal is converted to a mash state that facilitates compaction, *via* treatment with heat, water, pressure and time.[30] Conditioning is a prerequisite for the compression of meal into pellets.[25] Conditioning results in improved functional, nutritional and hygienic properties. Functional qualities affected by conditioning include pellet hardness and durability through the increase of material binding properties.[30] Both hardness and durability tend to increase with increased conditioning temperature. Starch gelatinisation is a major contributor to increased binding,[31] and this gelatinisation also results in greater starch digestibility.[29] Protein denaturation also occurs, and this is also believed to enhance its digestibility.[29] However, improvement in the ileal digestibility of lysine after heat treatment was shown by van Barneveld *et al.* to occur concurrently with a decrease in lysine utilisation, due to the Maillard reaction.[32] Specifically, while the lysine was digested, it was not in a form that could be utilised by the animal.

The temperature of conditioning has to be high enough to facilitate binding and destroy pathogens, while minimising destruction of protein. In general, meal reaches temperatures of approximately 80°C, with a steam pressure of about 440 kPa, and resides in the conditioner between 1 and 3 minutes.[29] The exact values depend mainly on the properties of the feed ingredients used.

Pelleting

In pelleting, the conditioned mash is forced through a compression die to produce pellets. This results in a further temperature rise, to reach between 86°C and 95°C. When the pellets have left the die they are not very durable and hence they are air cooled and dried before storage, to allow them to harden. At this point, heat sensitive ingredients, such as enzymes, may be sprayed on in liquid form. If loss of lysine due to Maillard reaction during

processing is economically significant, lysine and other amino acids could potentially be sprayed on at this point, as an alternative to current procedures. This technique does not appear to have been suggested or attempted previously, and is assessed in chapter 5.

4.1.3 The Maillard reaction during feed processing

The potential for the Maillard reaction to occur during feed processing has been acknowledged by researchers,[33,34] but few studies have investigated the extent of lysine loss during the pelleting of chicken feed. However, research has been undertaken to determine the nutritional impact of pelleting pig diets. Mavromichalis and Baker investigated lysine bioavailability after conditioning (60°C, 45 s), pelleting and storage of a complex nursery pig diet, and although they found no decrease in lysine availability after pelleting, an average of a 10% loss in lysine bioavailability was seen after storage of one week.[35] A study by Steidinger *et al.* found decreased weanling pig growth performance on feeds conditioned above 77°C (10 s).[36] The temperatures reached during the pelleting of poultry feed (80-90°C) are greater than during conditioning, in part due to the need to minimise microbiological contamination. These higher temperatures could be sufficient for the Maillard reaction to occur.[29] Higher processing temperatures are able to change the nutritional value of feed, as shown by van Barneveld *et al.*, who showed that heating field peas to 110°C for 15 minutes destroyed nutritionally available lysine by 4%, as shown by growth trials with pigs.[32] Peas do appear more resistant to heat damage than other feeds, such as soybean meal. This was shown by Hendriks *et al.*, who demonstrated losses of 4% in FDNB reactive lysine after extrusion of soybean meal at 93°C, 40% moisture.[34] The potential for damage to occur in feed ingredients prior to pelleting has also been established, for example, Rutherfurd *et al.* have shown the reduction in reactive digestible lysine in a variety of feed ingredients.[37]

4.2 PART I – MODELLING PELLET PROCESSING

The main purpose of this thesis was to determine whether the Maillard reaction occurs during feed pelleting to an economically significant degree. Ideally, this is done with growth trials; however this was expensive and unrealistic for initial testing. Pelleting trials

can also be expensive and difficult, as the smallest run on a typical industrial feed mill is 1 tonne. As outlined in previous sections, the pelleting procedure involves heat, steam and pressure. Hence, in the preliminary phases of the this work, autoclaving, which also uses heat, steam and pressure, was utilised as a cheap and easily accessible method of mimicking the pelleting process. Autoclaving has previously been used as a model of steam heating and commercial processing by Anderson-Hafermann *et al.*[38]

Using the standard run of the autoclave (wet heat sterilization of 20 minutes at 121°C with pressure at 10.3 kPa) the effect of autoclaving on barley flour by itself, in the presence of various carbohydrates, and with and without lysine was examined. Barley is an important ingredient in chicken feed, and was hence used as a protein source for these experiments. The degree of browning, the extent of protein crosslinking and the change in amino group content were all examined. Carbohydrates and lysine were included, to model what may occur under pelleting conditions. Finally, the protection of lysine with transglutaminase (TGase), an enzyme which catalyses crosslink formation between lysine and glutamine residues as discussed in section 4.3.4, was assessed.

Testing for lysine content becomes somewhat more difficult when samples contain both protein and free amino acids, as occurs in chicken feed where free amino acids such as lysine, methionine and threonine are often added. The ε-amino group of lysine residues, the α-amino groups of free amino acids and N-terminal protein ends will all react under assay conditions (Figure 4.2-a). Hence, all lysine assay results described in this chapter refer to the number of free amino groups present.

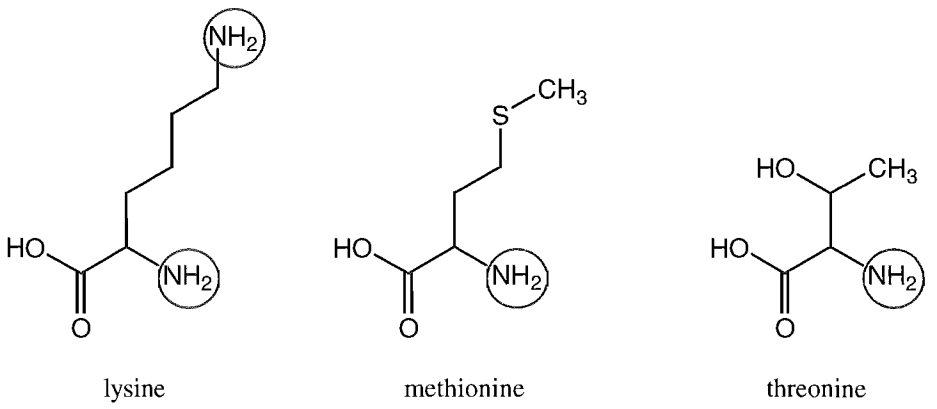

Figure 4.2-a: The structure of the free amino acids lysine, methionine and threonine. Amino groups that react under test conditions are circled.

4.2.1 The reaction of barley flour with carbohydrates during autoclaving

When barley flour was autoclaved without added carbohydrate, the constituent proteins underwent a small amount of crosslinking, as visualised by smearing in SDS-PAGE (Figure 4.2-b), and a concurrent 22% reduction in amino group content (Figure 4.2-c). When carbohydrate was added, the effect depended upon the carbohydrate type. Five different carbohydrates: xylose, molasses, starch, malt extract and dextrin, which were described in chapter 3, were tested for reaction with barley flour under autoclave conditions.

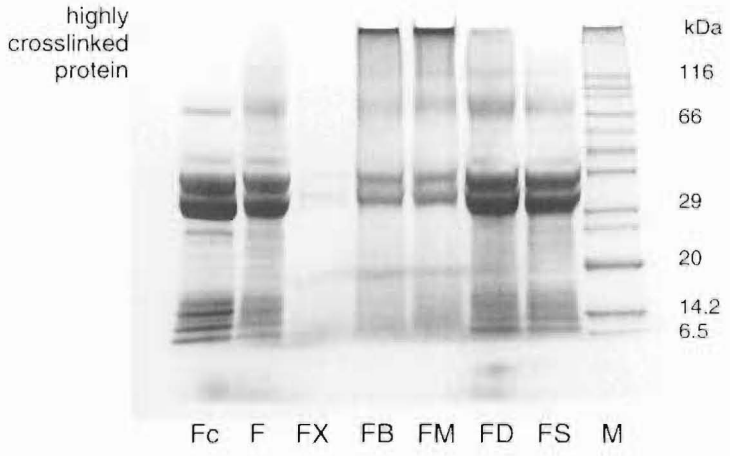

Figure 4.2-b: SDS-PAGE showing the degree of crosslinking in barley flour total protein extracts after autoclaving with various carbohydrates. Fc: control flour (not autoclaved); F: flour; FX: flour + xylose, (2:1 w/w); FB: flour + molasses, (2:1 w/w); FM: flour + malt extract, (2:1 w/w); FD: flour + dextrin, (2:1 w/w); FS: flour + starch, (2:1 w/w); M: Sigmamarker (wide-range).

Xylose is known to be reactive with proteins,[39] and its crosslinking and amino group reactivity was characterised extensively in chapter 3. It is also a pure reducing sugar, unlike the other carbohydrates used, and hence was expected to be the most reactive, as shown in chapter 3. This occurred, as shown by SDS-PAGE (Figure 4.2-b), where no protein bands were visible after reaction of barley flour with xylose, due to the large degree of crosslinking that occurred. Correspondingly, a large reduction in amino group content (69%) was measured in this sample (Figure 4.2-c).

Molasses and malt extract were the next most reactive, as shown by SDS-PAGE (Figure 4.2-b), which was consistent with data shown in chapter 3. On reaction with malt extract, barley flour proteins showed a 74% reduction in amino groups. It was expected, from the comparable SDS-PAGE results, that the molasses sample would show a similar loss. However, autoclaving flour with or without molasses did not lead to significantly different amino group contents (Figure 4.2-c). This is consistent with results from RNase A incubations with molasses, where large increases in amino group contents were seen after incubation of RNase A with molasses for 6-8 days at 37°C. This suggests that the molasses also caused the protein in the barley flour samples to fragment, resulting in a higher than expected amino group reading.

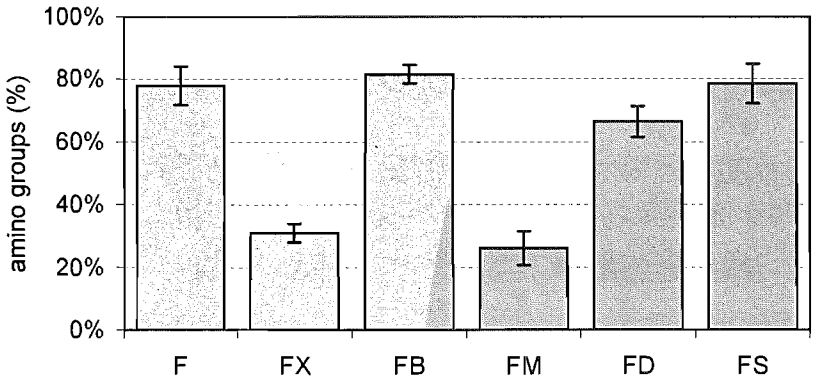

Figure 4.2-c: Percentage change in lysine content (measured using the mOPA method) of barley flour total protein extracts after autoclaving with various carbohydrates. F: flour; FB: flour + molasses, (2:1 w/w); FS: flour + starch, (2:1 w/w); FD: flour + dextrin, (2:1 w/w); FM: flour + malt extract, (2:1 w/w); FX: flour + xylose, (2:1 w/w). All data are shown relative to the control (not autoclaved) flour. Error bars represent standard error of triplicate measurements.

Starch, which would not be expected to have significant impact on crosslinkage or amino group loss due to the few reducing ends it contains, behaved as expected, with amino group loss similar to that found with no added carbohydrate. Again, this was consistent with results found in chapter 3, after incubation with RNase A. Dextrin, a partially hydrolysed form of starch, showed slightly higher reactivity than starch by both SDS-PAGE

and amino group loss, as would be anticipated from the increased number of reducing ends present after partial starch hydrolysis.

Overall, it is apparent that the addition of reducing sugars to barley flour increases the rate of crosslinking and amino group modification that occurs under autoclave conditions. It is also important to note that crosslinking and amino group loss did occur to a lesser extent in the barley flour samples that contained no added sugar, indicating that the reducing carbonyl groups present naturally in the flour were able to react under the conditions used. Alternatively this could be general aggregation, as noted in RNase A controls incubated at 70°C (chapter 3). However, the potential for the Maillard reaction to occur under feed processing conditions was clearly demonstrated.

Addition of free lysine to autoclaved samples

As lysine is commonly added to feed meal prior to processing, trials were performed with lysine added to flour and xylose mixtures, to see what impact this would have on Maillard reaction. Both the effect on browning and on amino group loss was measured, on samples with and without a reducing sugar (xylose). As can be seen from Figure 4.2-d, the addition of lysine to an autoclaved flour sample, or a flour/xylose sample increased browning markedly. This would be expected, as many more amino groups were available to react, leading to the brown advanced Maillard products.

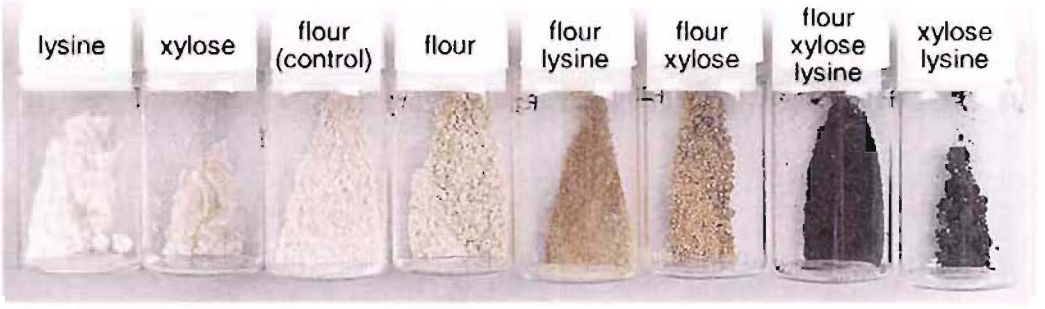

Figure 4.2-d: Barley flour samples showing various degrees of browning after autoclaving with and without xylose and lysine. Flour (control) was not autoclaved, all other samples were autoclaved. In flour samples, where present, xylose was added at 5% and lysine at 1%. In xylose/lysine sample, the ratio was 1:2 (w/w). All percentages calculated by weight.

The degree of crosslinking for samples containing lysine is illustrated in the SDS-PAGE gel shown in Figure 4.2-e, where it can be seen that the addition of lysine to the flour/xylose sample reduced the disappearance of flour protein bands slightly, and increased the presence of small protein fragments, indicating a reaction between xylose and free lysine, as expected. In the sample containing only xylose and lysine, a large degree of reaction occurred forming higher molecular weight melanoidins (6-29 kDa), which conjugated with Coomassie Brilliant Blue stain to be visible by SDS-PAGE.

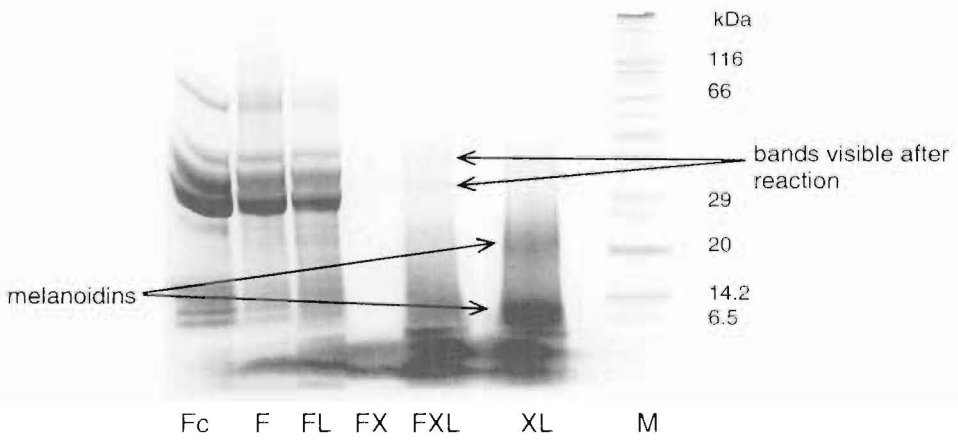

Figure 4.2-e: SDS-PAGE showing degree of crosslinking in barley flour total protein extracts after autoclaving xylose and lysine. Fc: flour control; F: flour; FL 99% flour, 1% lysine; FX: 95% flour, 5% xylose; FXL: 94% flour, 5% xylose, 1% lysine; XL: 33% xylose, 67% lysine; M: Sigmamarker (wide-range). All percentages calculated by weight.

Analysis of these lysine containing samples by the mOPA method (Figure 4.2-f) showed that in most samples, reduction in amino group content had occurred in a manner predictable from the experiments detailed above. Addition of xylose resulted in a decrease in the reactive amino groups present in the samples, to a slightly lower degree than seen in the previous sample containing more xylose. The lysine, in the xylose/lysine system, reacted to reduce the amino group content by 53%. However, amino groups were present in a large excess in this sample, and on an equimolar basis only 30% of the amino groups present would have been expected to react. Therefore, amino groups may have also been reacting further with Maillard reaction products, or been subject to degradation during heating.

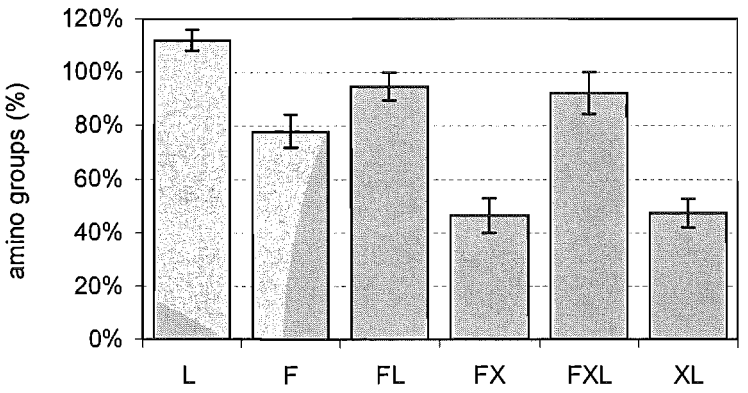

Figure 4.2-f: Typical amino group content (measured using the mOPA method) of barley flour total protein extracts after autoclaving with xylose and lysine. L: 100% lysine; F: 100% flour; FL 99% flour, 1% lysine; FX: 95% flour, 5% xylose; FXL: 94% flour, 5% xylose, 1% lysine; XL: 33% xylose, 67% lysine. All data are shown relative to a non-incubated control of the same composition. Error bars represent standard error of 3 measurements.

4.2.2 Summary of results

Significant losses of amino groups occurred, with concurrent formation of crosslinks, when barley flour was autoclaved by itself. This loss of reactive amino groups was accelerated significantly by the addition of xylose and malt extract, and moderately by the addition of dextrin. However, as expected, the addition of starch did not alter the reaction significantly. The autoclaving of molasses with barley flour resulted in increased formation of crosslinks, as compared to standard flour, but did not significantly reduce the amino group content, relative to a flour sample without added carbohydrate, suggesting protein fragmentation. This suggests that starch and dextrin are much better feed additives than molasses and malt extract. The addition of lysine to the flour, and flour/xylose mixtures, appeared to result in lower percentage lysine being lost during processing in general, although greatly increased levels of browning were seen.

These results show that the Maillard reaction does occur in barley flour under the conditions of autoclaving. This indicates that there is potential for bioavailable lysine loss

to occur under the conditions of processing meal into pellets. Based on these results, Tegel Foods Ltd. agreed to perform pelleting trials at their feed mill in Christchurch, New Zealand.

4.3 PART II – ANALYSIS OF LARGE SCALE PROCESSED FEED

Prior to pelleting trials being performed at the Tegel feed mill, as detailed in section 4.3.2, an initial visit to the feed mill was made and samples were collected by operators at the feed mill at 3 points during a standard feed run – the unprocessed meal, the feed as it left the conditioner (ex-conditioner) and the final pellets. Sample preparation and testing methodology was optimised on these three samples. The composition of the basal diet utilised is shown in Table 4.3-a.

Ingredient	Content (%)
wheat (NZ)	25.00
barley (NZ)	41.10
soybean meal	18.20
meatmeal	11.50
tallow	2.80
L-lysine-HCl	0.340
methionine	0.310
threonine	0.075
sodium bicarbonate	0.290

Table 4.3-a: The main ingredients in the Tegel Broiler finisher 1 (F1) feed at the time of the trials.

4.3.1 Sample preparation

The main aim of initial testing with the three samples of chicken feed was to minimise variation between different extractions. Various methods of sample grinding and protein extraction were tested in order to determine the effect of these on sample uniformity.

Grinding

Initial grinding of samples was performed using a 1 mm mesh. The standard error for lysine quantitation of the meal and ex-conditioner samples, irrespective of extraction technique, was approximately 7%. For the pelleted samples, presumably due to the homogenising action of pelleting, this standard error reduced to approximately 2%. In order to reduce this error, samples were ground to pass through a 0.5 mm mesh. This reduced the variation in the meal and ex-conditioner samples considerably – to approximately 1%. Therefore, all further samples were extracted after grinding to a maximum diameter of 0.5 mm.

Extraction

A variety of conditions were used to extract protein and amino acids from the chicken feed. From this it was found that the most important factor in gaining consistent results was performing all extractions at the same time, or alternatively to extract a reference control sample with each batch, as suggested by Friedman for the ninhydrin method.[40] It was interesting to note that some proteins, in the 45-66 kDa range, appeared to extract into water more efficiently than into the standard extraction solution (Figure 4.3-a). However, as would be expected given the lack of reducing agent in water, a major protein band at about 29 kDa extracted into standard solution to a far greater degree than into water.

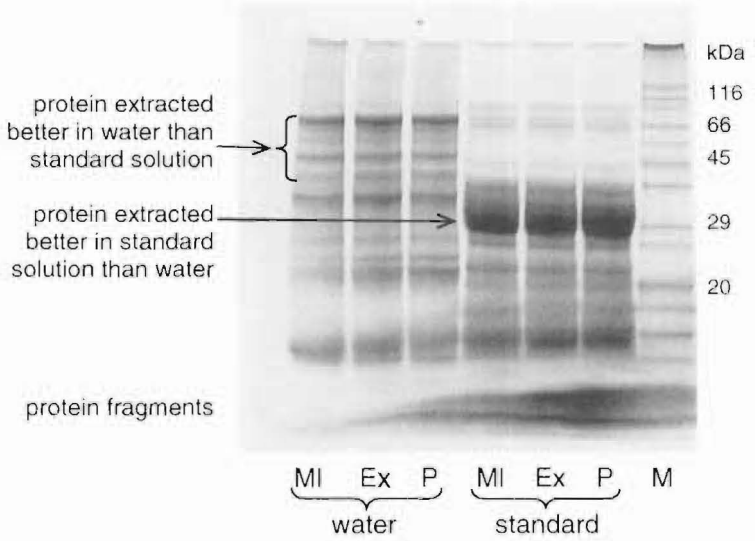

Figure 4.3-a: Typical (reduced) SDS-PAGE of proteins extracted from chicken feed into water or the standard extraction solution (50% 1-propanol, 1% DTT) from Ml: meal; Ex: ex-conditioner; P: pellets.

Figure 4.3-b shows the average amino group contents of meal, ex-conditioner and pellet samples after extraction by a variety of methods. Virtually all extraction techniques gave equivalent results when the changes in percentage amino group contents were compared. The major importance of these results was that they showed clear loss in the number of reactive amino groups in the samples as a result of processing. A drop of 18% between the meal and the ex-conditioner samples was apparent.

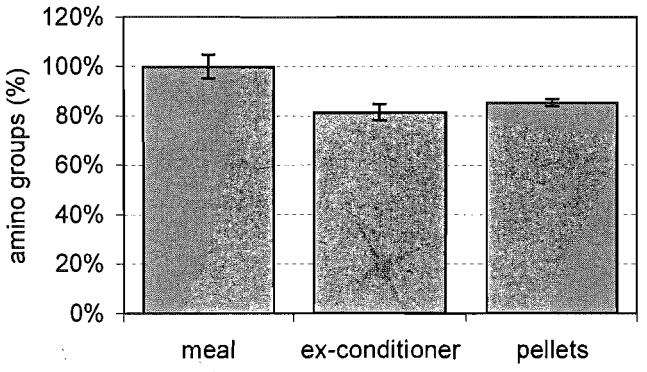

Figure 4.3-b: Average values of amino group contents, displayed as percentage of the meal sample. Error bars represent standard error of 8 replicate extractions.

The lack of decrease between the ex-conditioner and pellet samples was indicative of proteins fragmenting to produce reactive N-terminal amino groups as a result of the forces applied during the pelleting stage of processing, masking any potential reduction in reactive amino groups that occurred at this stage of the processing. Protein fragments were obvious by SDS-PAGE (Figure 4.3-a), and the degree of fragmentation was consistently greater in the ex-conditioner and pellets samples, consistent with protein fragmentation occurring as a result of processing. Therefore, the overall reduction in reactive amino groups throughout the processing procedure may have been much higher than the 15% approximate difference seen between the amino group contents of the meal and the pelleted feed. Consistent with amino group modification occurring to a greater degree in free amino acids, no crosslinking was apparent in proteins extracted from the pellet samples (Figure 4.3-a).

As the pelleting stage involves greater pressure and a higher temperature than the conditioning stage, it would not seem unreasonable to assume, as during conditioning, an 18% loss could have occurred during the pelleting step to give a total reduction in reactive amino groups of 36% or higher. This result was very significant, as it not only indicates the potential for the Maillard reaction to occur during the processing of chicken feed, but shows that the reduction that occurs in reactive amino groups could be very large, and hence of economical significance. The reaction blocking the amino groups could have occurred at any of the amino groups present in the protein or the free amino groups added to the feed. As free lysine has two free amino groups per molecule, comprising about 37 of the 65 mmol/kg DM amino groups present as a result of free amino acid addition, this was probably the amino acid most affected.

4.3.2 The effect of removing selected free amino acids from the meal

Based on the results above, the Christchurch Tegel Feed Mill performed trial feed runs with selected amino acids removed from the meal, to determine the effect this would have on the free amino group content of the meals, ex-conditioner samples (collected on exiting the conditioner), and pellets. This involved a large commitment from Tegel, as the minimum feed run is 1 tonne. Operators at the feed mill undertook to alter the feeds on my behalf, and to collect samples from different stages of the process.

Four sets of samples were taken, one from a standard run, and one set each from runs with lysine, methionine or threonine removed. These were the only amino acids added to the feed meal. Unfortunately, due to circumstances beyond my control, no trial feed run was performed where all amino acids were removed.

The standard feed meal was compared to the altered feed meal to gain an indication of the error introduced by different feed runs, and from the testing method used. This comparison is shown in Figure 4.3-c, where the concentration of amino groups found for each meal was compared with theoretical values calculated from the formulation provided for the standard meal. It is assumed that standard meal contains precisely the amount of free amino acids stated in Table 4.3-a.

These results indicated a higher than expected concentration of amino groups in the samples with methionine and threonine added, or only lysine added. In contrast, the samples with lysine and methionine, and with lysine and threonine had very good correlations with the theoretical concentration of amino groups. A partial explanation is that sampling error may have been introduced, as the sample collection procedures at the feed mill were performed without my observation or control, due to technical constraints.

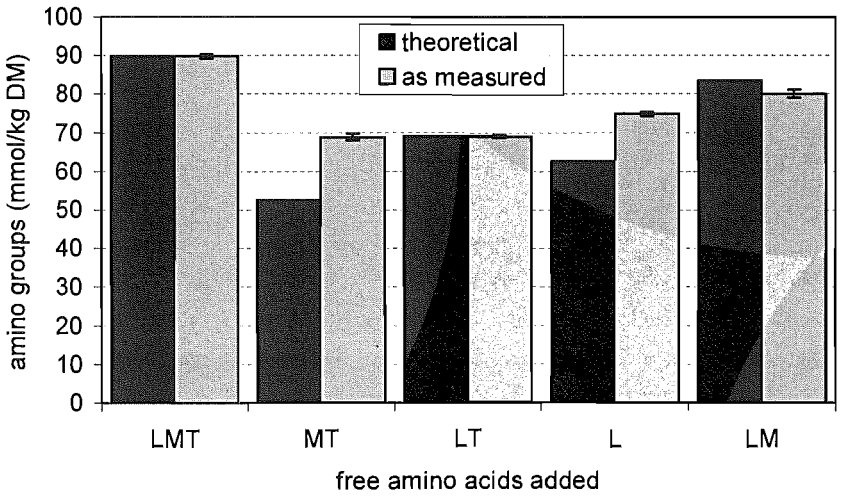

Figure 4.3-c: The amino group content of the trial feeds compared with the theoretical content. The theoretical amino acid content was calculated by estimating the base amino acid content of the meal from the standard feed, and adding the amount of free amino groups contributed by free amino acids in each trial feed. L: lysine; M: methionine; T: threonine. Error bars represent the standard error of ten replicates extraction for each meal sample.

In Figure 4.3-d, the results for percentage amino group contents, relative to the respective meal sample, are shown for all trial feed runs. These show that no consistent pattern of amino group reduction as a result of processing. However, the feeds without lysine added (MT), with only lysine added (L), and with all amino acids added (LMT) did show a decrease between either the meal and the ex-conditioner, or the meal and the pellets.

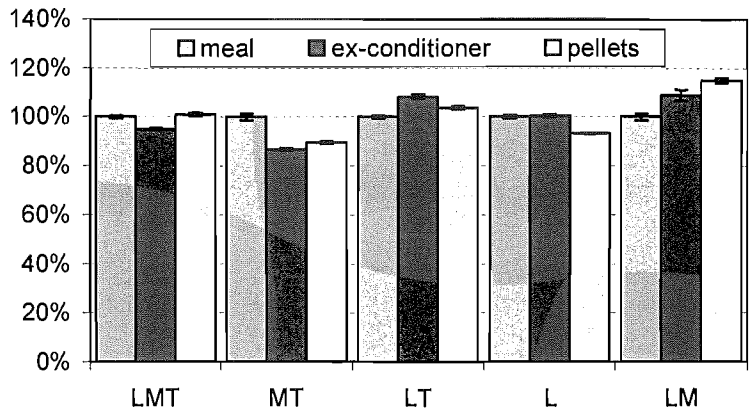

Figure 4.3-d: Percentage amino group contents, measured using the OPA method, of samples taken from the meal, ex-conditioner and pellet stages of processing for runs performed with trial feeds with various free amino acids added. Values calculated relative to the respective meal sample. L: lysine; M: methionine; T: threonine; LMT represents standard feed. Error bars represent the standard error of 10 replicate extractions.

In Figure 4.3-e, the changes in amino group content brought about by processing are analysed. If amino groups had reacted *via* the Maillard reaction during processing, a negative change would be expected, whereas a positive change would be expected if the shear forces present during processing had broken up the protein to produce more N-terminal amino groups. Both negative and positive changes were seen, suggesting that both of these processes were working in concert. This is in concurrence with the results from the initial feed samples (section 4.3.1), where a decrease was seen between meal and ex-conditioner, but an increase was seen between ex-conditioner and pellets.

While it is possible that a degree of the variation in these samples was due to collection techniques, these results do indicate the potential for substantial lysine loss, of at least 5-10%, to occur during processing. However, this loss could be far greater, due to the counteracting effect of the formation of amino groups through the fragmentation of proteins. This reduction in amino group content during these trials was not to the degree seen in the first sampling, however. This indicates that different runs at the feed mill could

have large effects on the reactive amino group contents of the samples, and that careful monitoring of the pellet manufacture could have a large impact on the consistency of nutritional quality in the resulting feed. Further work could investigate the most suitable techniques for testing this variation during production runs on a regular basis.

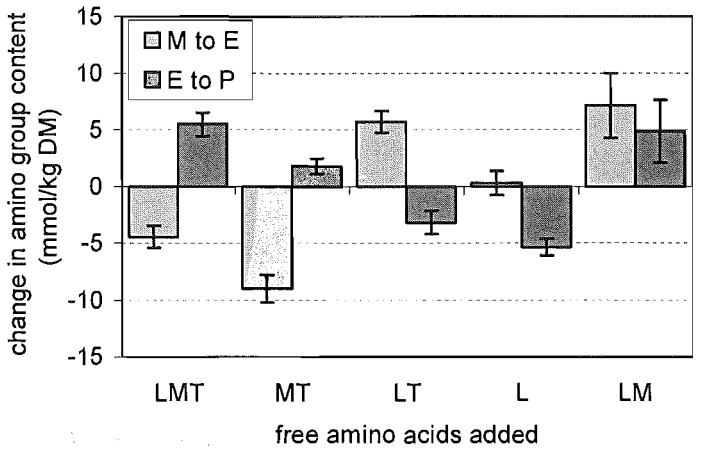

Figure 4.3-e: Change in amino group concentration between the meal and ex-conditioner (M to E) and ex-conditioner and pellets (E to P) for each of the trial feeds. Error bars represent total standard error for meal plus ex-conditioner, or ex-conditioner plus pellet, samples for each trial diets.

4.3.3 Summary of results

The results found by analysing chicken feed at different stages of processing indicates that amino groups can react during the pelleting procedure, but it was difficult to estimate the degree of loss due to an increase in free amino groups from the shearing of proteins during the processing. This reduction could potentially be 36% or higher, based on the results observed here. It is difficult to predict the roles that each of the free amino acids play in this process, due to the shearing effects of the processing, and because of the variation between feed mill runs. This difference is presumably due to the inherent nature of the procedure, which may have inter-run variations in humidity and temperature.

Furthermore, variations in different batches of grains, and other ingredients, may alter the results found. For example, the amino acid digestibility of meat and bone meal can be highly varied, with reports of lysine digestibility ranging between 45% and 86% in just 21 different samples.[41] However, as the free amino groups present in the lysine contributes almost half of the amino groups present in the feed samples, it is most likely that this was the amino acid most affected during processing.

Even a small reduction in the concentration of nutritionally available lysine is of significant economic consequence to the poultry industry. In addition, even a small amount of Maillard reaction may result in products with antinutritional properties.[42] While there is no direct evidence that Maillard products have an antinutritional effect in poultry, evidence from rat trials indicates this is possible. Therefore Tegel Foods Ltd. undertook a growth trial to determine the extent of this lysine loss and to establish whether it was of economic concern. This is outlined in Chapter 5.

4.3.4 Potential methods of minimising lysine loss during processing

If the potential nutritional loss of lysine during processing is to be more than an interesting addition to our knowledge, methods of preventing or minimising this loss have to be found. To be relevant to the poultry industry, the method used must be a simple, cheap alteration to the currently used process. Two potential approaches to this lysine loss minimisation were explored: TGase protection of lysine residues, or adding the lysine post-pelleting.

TGase protection

Transglutaminase (protein-glutamine γ-glutamyltransferase) (TGase) is an enzyme which is found in many organisms, including the human body,[43] where it is responsible for the formation of hard blood clots by catalysing the formation of covalent crosslinks between lysine and glutamine residues (Figure 4.3-f).[44] More recently, TGase has been utilised in food processing.[45-48] The new crosslink formed is believed to not only prevent lysine from

taking part in the Maillard reaction, but also to leave lysine nutritionally available.[49] Hence, there is potential for TGase to play a role in the protection of lysine during feed processing.

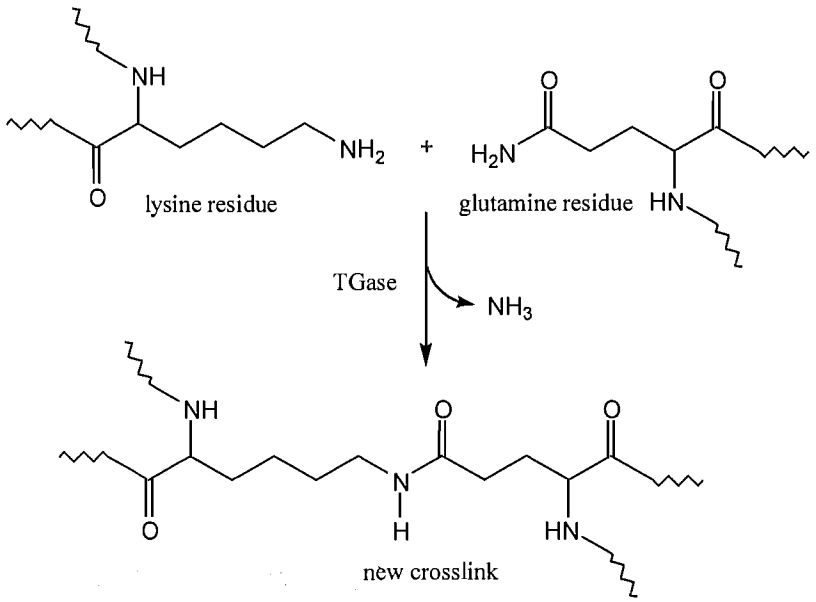

Figure 4.3-f: Transglutaminase mediated formation of a crosslink between lysine and glutamine residues.[53]

Recently, TGase has become commercially available from a microbial source, making it more viable as a food or feed additive. It is also already approved as a food additive, and is currently used as an improver of the functional qualities of meat products. Work by Gerrard *et al.*[50] and Rasiah[51] have shown that TGase reacts virtually immediately with the proteins in bread dough, indicating that it may work on a viable timescale to be used in feed pelleting. A study by Basman *et al.* has shown that barley has a similar reactivity with TGase to wheat, whereas soy proteins showed higher reactivity,[52] hence these ingredients could also be protected during pelleting by the use of TGase.

Preliminary work was undertaken, using autoclaving of flour, carbohydrate and lysine samples with and without TGase. Under these conditions, it did not appear that TGase had any large protective effect (Figure 4.3-g), despite incubation at room temperature for 2 hours prior to autoclaving. In Figure 4.3-g, no obvious difference can be seen between

samples autoclaved with TGase, and those that were not. If protection had occurred, it would be expected that the samples containing xylose and TGase would have shown a greater amount of protein on the gel, similar to that seen for the samples without xylose. Results from lysine count results also showed no significant differentiation between samples with and without TGase. As TGase has been shown to be active with barley flour previously,[52] this may have been due to insufficient moisture in the samples to allow this enzyme catalysed reaction to occur. Thus, use of TGase in the context of protecting lysine in chicken feed was not further explored.

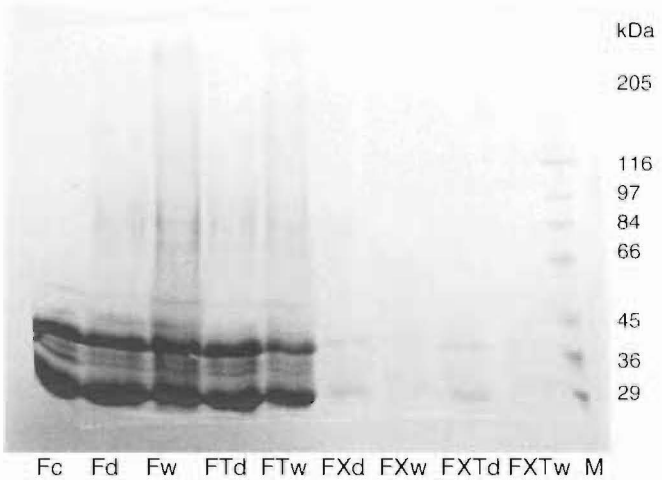

Figure 4.3-g: SDS-PAGE showing proteins extracted from barley flour after autoclaving with or without xylose, TGase and water. Fc: control flour (not autoclaved); Fd: flour without water; Fw: flour and water; FTd/w: flour and TGase, with and without water; FXd/w: flour and xylose, with and without water; FXTd/w: flour, xylose and TGase, with and without water; M: Sigma wide-range marker.

Application of free lysine post-pelleting

Some antibiotics and enzymes, which have been shown to lose activity on processing, are added post-pelleting.[54] It was determined that spraying a lysine solution on to the pellets was a potential method for adding free lysine to feed in such a manner as to prevent its loss as a result of processing. This option is both economically and technically realistic, and is studied in chapter 5.

4.4 CONCLUSIONS

There is potential for significant amounts of lysine to be lost during processing, but due to dual processes of lysine lost through Maillard reaction, and terminal amino groups gained through shear processes, the degree and significance of this loss is difficult to determine without growth trials. The free lysine in the pellet could be prevented from undergoing Maillard reaction if applied post-pelleting. On these grounds, Tegel Foods Ltd undertook a growth trial to clarify the significance of the Maillard reaction during feed pelleting.

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LYSINE LOSS DURING FEED PROCESSING: BROILER GROWTH TRIALS

CHAPTER 5

5.1 BACKGROUND

As reported in chapter 4, chicken feed was analysed at pre, mid and post processing stages. The results from these studies indicated that there was potential for lysine to be lost during the pelleting procedure. Consequently, a growth trial was conducted with the aim of determining whether a significant reduction occurs in the levels of reactive lysine as a result of Maillard reaction occurring during processing, and whether it would be more effective to add free lysine in liquid form, post-pelleting, *via* method (ii) as outlined in Figure 5.1-a.

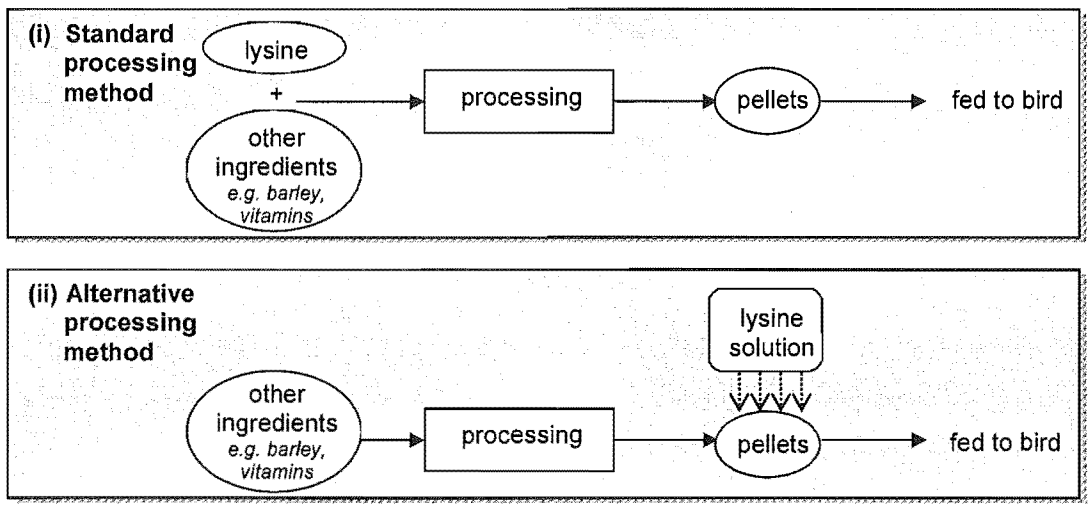

Figure 5.1-a: Two methods for adding lysine to feed. i) The standard method of adding lysine to the other ingredients prior to pelleting; ii) the alternative method of spraying lysine, in solution, on to the outside of the pellets.

5.2 GROWTH TRIAL

The main purpose of this growth trial was to determine the influence of pre- or post-pelleting additions of lysine on the performance of broiler chickens. This trial would also determine how well feed processed with lysine supported bird growth, and hence how much lysine was lost through processing due to the Maillard reaction. Additionally, the trial would provide an excellent reference of how well the lysine values given by the OPA method compared with the growth of chickens in the trial.

5.2.1 Trial design

The purpose of this growth trial was to determine the relative growth supported by a series of diets that varied in lysine content. Over the course of the trial, growth, feed intake and mortality on each diet were recorded. From these, the total growth supported by each diet, and the efficiency of the conversion of feed to weight gain (FCR, as described in chapter 4), were determined.

The growth trial was designed with 8 diets. The reference diet (Diet 1) was a maize/soy F1 formulation from the Tegel feed mill in New Plymouth, supplemented pre-pelleting (Figure 5.1-a(i)) with an amount of free lysine anticipated to be sufficient for optimal bird growth. An external reference wheat/soy based diet from the Christchurch feed mill (Diet 2), which had been similarly supplemented with lysine pre-pelleting, was also included. Experimental Diets 3-8 were based on the New Plymouth finisher 1 (F1) maize/soy based formulation, unsupplemented by free lysine. These diets were then supplemented, post-pelleting (Figure 5.1-a(ii)), with free lysine at the levels indicated in Table 5.2-a. The free lysine was applied to the outside of the pellets by first dissolving an appropriate amount of lysine-HCl into 1 litre of water, then spraying this solution onto the pellets as they were mixed in a rotary mixer. Once sprayed with lysine, each diet was bagged and stored in a warehouse for one week, until the start of the trial.

Diet	Diet base	lysine-HCl added (g/kg)	
		pre-pelleting	post-pelleting
1	New Plymouth Standard F1 *(reference diet)*	4.40	-
2	Christchurch Standard F1 *(external reference diet)*	3.90	-
3	lysine deficient NP std F1	-	-
4	lysine deficient NP std F1	-	0.88
5	lysine deficient NP std F1	-	1.75
6	lysine deficient NP std F1	-	2.62
7	lysine deficient NP std F1	-	3.50
8	lysine deficient NP std F1	-	4.37

NP std F1: New Plymouth standard F1

Table 5.2-a: Theoretical lysine contents of the diets for the Tegel growth trial.

The growth trial was designed with 8 replicates of 7 male birds on each of the treatments (Diets 1-8). Chicks (600) arrived at the trial facility within 24 hours of hatching, and were fed on a standard starter diet for their first week.

At day 8, the chicks were weighed and the mean weight determined. Of the 600 birds, 25% with weights furthest from the mean were discarded from the trial. The remaining 448 birds were allocated into 64 groups of 7 birds, such that average gross weight of each group was 1272 g, with a range of 2%. Trial diets were randomly allocated to groups of birds, to control for position in the trial facility and associated variables. Feed was available *ad libitum*, with a set weight available per group. Bird mortality was checked on a daily basis, and the dead weight recorded. At the ends of the first and second weeks on the trial diets, the weight of each cage of birds was measured and recorded. At the same time, the weight of uneaten food in each trough was recorded. Growth, feed intake, FCR and mortality data from this growth trial is presented in Appendix II.

The timeline of the growth trial is outlined in Figure 5.2-a. This diagram also shows the points when samples were collected for later lysine analysis, as detailed in section 5.3.

During week 1 of the growth trial, birds are aged 8-14 days and during week 2, birds are aged 15-21 days. Therefore, for clarity of discussion, the growth trial weeks are referred to as week A and week B respectively, where bird age is day 8-14 and day 15-21 respectively.

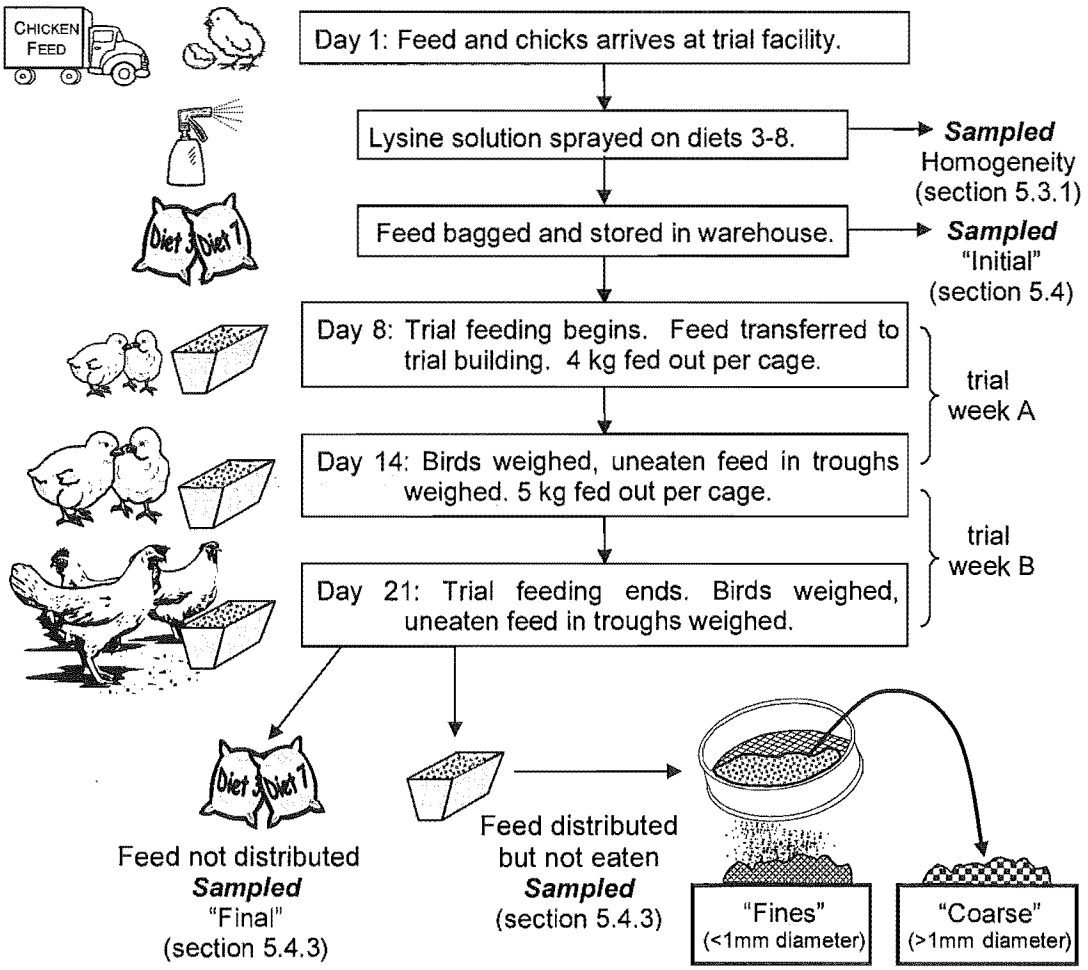

Figure 5.2-a: Schematic of the timeline of the growth trial, detailing the points where samples were taken for lysine testing. Sampling was performed for each diet at each sampling point. For the feed that was distributed but not eaten, for each diet samples were collected from the 8 feed troughs containing that diet, and pooled.

5.3 LYSINE ANALYSIS

There were three main reasons for conducting lysine analysis of the trial diets. Firstly, measured lysine contents were required to determine how well measured lysine content compared to theoretical lysine content, and to bird performance. If Maillard reaction had occurred during processing, Diet 1 would show a lower lysine content, as measured by the OPA method and assessed by bird growth, than expected from the theoretical amount of added free lysine. In conjunction, Diets 3-8 were designed to show the effectiveness of the alternative method of adding lysine post-pelleting. Additionally, by comparing measured lysine contents with bird performance on all diets, the relevance of the lysine content data quantified by the OPA method to that of an *in vivo* environment could be assessed.

Secondly, the level of lysine in the diets at the time of lysine addition ("Initial") was compared with that on the final day of the growth trial ("Final") (Figure 5.2-a). This was required to determine whether lysine may have reacted *via* the Maillard reaction during the storage of the feed. Feed distributed in the final week had been stored in a warehouse for two weeks and in the trial facility with the chickens in the final week, at around 29°C. Significant reduction in lysine has been shown to occur in nursery pig diets under similar conditions,[1] and hence had to be assessed in these samples.

Finally, samples were taken from the feed remaining in the troughs at the end of the trial and separated into fine ("Fines") and coarse ("Coarse") components for each diet (Figure 5.2-a). Pellets are susceptible to crumbling over time, and durability is one of the major tests of pellet quality.[2] As a result, when lysine is sprayed on to the outside of the pellet there exists potential for significant quantities of this lysine to be included in the material that has fallen off the pellet. Birds prefer to eat pellets over this fine material,[3] and hence may have a lower lysine intake if a large proportion of the lysine is in the fine material. Therefore, these fine and coarse samples were analysed for lysine content to determine whether significant quantities of lysine had fallen off the outside of the pelleted Diets 4-8, and remained in the uneaten fines. From this, it could be established if any significant difference existed between the diets given, and the diets eaten by the birds over the course of the experiment.

For the purposes of this study, the major point of interest was lysine loss from the added lysine, as this can be added post-processing. As discussed in chapter 4, where protection of flour proteins using TGase was attempted, it is much more difficult to protect the lysine residues in the protein of other feed ingredients from the effects of processing. Hence, these protein-bound lysine residues were of reduced importance in the bounds of this growth trial. Hence, for lysine analysis purposes, it was only the added free lysine that was of relevance. Therefore, it was decided that performing the standard OPA method on water extracts of the samples would be preferable and all free lysine was assumed to be easily solubilised into this medium. Other added amino acids, such as threonine and methionine would also be soluble, as would a small proportion of the other proteins. These should be equivalent for all samples from the New Plymouth feed mill, and therefore could be accounted for in calculations. However, it was important that all samples sets received equivalent treatment during extractions to ensure that this base level remained unchanged.

Therefore, samples collected, as detailed in Figure 5.2-a, were ground finely, followed by water extraction and lysine analysis of this extract. For each sample, multiple extractions were performed at the same time and each extract was tested in triplicate, unless otherwise noted, for lysine content by the OPA method. The term 'replicate' used in the following analysis refers to the number of these multiple extractions. For comparison with the amount of added free lysine, amino group contents were calculated as lysine equivalents by comparison with a lysine calibration curve (chapter 2), and are shown in the following analysis as grams of lysine per kilogram of dry matter (DM).

5.3.1 Diet homogeneity

Free lysine was added post-pelleting in Diets 4-8, therefore the homogeneity of the diets after post-pelleting lysine addition had to be assessed to ensure the diets being eaten by the chickens had little variation within each trial group. To measure the homogeneity of the feed, six samples of Diet 6 were taken from different areas in the rotary mixer immediately after the lysine solution had been sprayed on. On each of the six samples, six

extractions were performed, and each of these was assayed in duplicate for lysine. The results were averaged for each sample, and are shown in Figure 5.3-a.

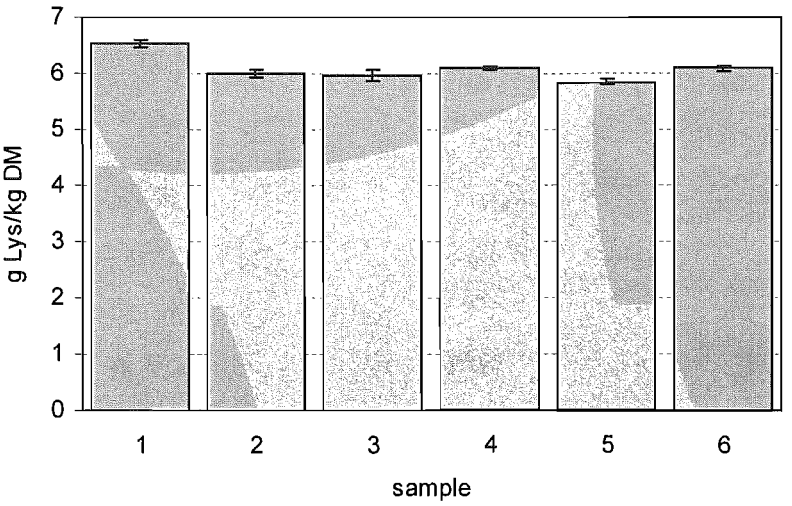

Figure 5.3-a: Bar graph showing variation in lysine content of six samples taken from Diet 6 immediately after the lysine solution had been sprayed on. Error bars represent standard error of 6 replicates.

From these results it can be seen that five of the six samples have very similar lysine contents, although sample 1 was significantly higher, but only by 10%. It was decided that this level of homogeneity was satisfactory for single sample analysis of each diet, and that all of the birds on the same diet would be receiving equivalent levels of lysine.

5.4 LYSINE CONTENTS OF DIETS 1-8

As the homogeneity of the diets had been established as satisfactory, lysine content analysis of all diets was performed.

5.4.1 Initial samples compared with final samples

From Figure 5.4-a, it can be seen that no loss of reactive lysine occurs during the storage of the feed over the course of the growth trial. In five diets, the level of lysine measured was actually higher at the end of the trial, which may have been due differences in base amino group extraction level, as discussed in chapter 4. Hence, for each diet, the "Initial" and "Final" lysine count data were averaged, and the averaged numbers were used in further analysis (referred to as "As Fed").

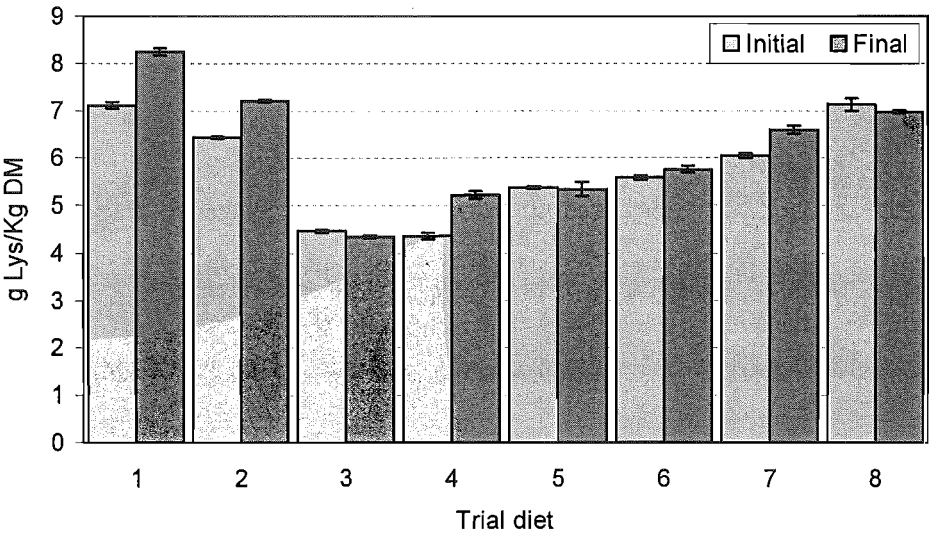

Figure 5.4-a: Lysine contents for Diets 1-8 for samples taken at the time that lysine was added to Diet 4-8 (Initial) and as fed on the last day of the growth trial (Final). Error bars represent standard error of 5 replicates.

5.4.2 Comparison between measured lysine content and theoretical lysine contents for Diets 1-8

To compare the amount of measured lysine, in grams of lysine per kilogram of dry matter, with the amount of lysine added, in grams of lysine per kilogram of feed, two factors must be taken into account. The first factor was that the 'mass of lysine added' was given by

Tegel for the lysine-HCl salt (MW 182.6), whereas measured lysine content was for the free lysine moiety (MW 146.19). The adjustment factor for this was 0.80. The adjusted values are shown in Table 5.4-a. The second factor to be accounted for was the moisture content of the trial feeds. The moisture content was different for each sample series collected, with the average percentage moisture as follows: Initial 9.2%; Final 8.9%; Coarse 8.3%; Fines 7.9% (average standard error, for each set of 8 replicates, of 0.08%). To make all results comparable with the mass of lysine added to the feed (moisture content 9.2%), all results were divided by (100% - 9.2%). The results of this adjustment for the "As Fed" samples are also shown in Table 5.4-a.

	Diet	1	2	3	4	5	6	7	8
Mass added (g/kg) (theoretical)	*Lysine-HCl*	4.40	3.90	0	0.88	1.75	2.62	3.50	4.37
	Lysine	3.52	3.12	0	0.70	1.40	2.10	2.80	3.50
Measured lysine content	*g/kg DM "As Fed"*	7.68	6.82	4.42	4.80	5.35	5.67	6.32	7.05
	g/kg ("As Fed" adj)	8.46	7.51	4.86	5.28	5.89	6.24	6.96	7.77

Table 5.4-a: Adjusted values[a] for mass of lysine added as a solution to Diets 1-8 and for measured lysine contents for Diets 1-8 as fed, adjusted for moisture content. DM: dry matter.

The adjusted "As Fed" results for Diets 1-8 are graphed against the lysine addition values in Figure 5.4-b. The correlation between added lysine and measured lysine indicated good methodology for the lysine assay. It should be noted that the slope of the correlation was lower than expected, with 0.81 gram of lysine measured for every gram of lysine added. This would indicate that for each addition amount, approximately 19% of the added lysine

[a] These values do not equate to the total lysine contents of the feeds, which would be expected to be at least 13 grams of lysine per kilogram of feed for Diets 1 and 2. The values shown are much lower as water extraction does not extract all lysine in the samples. In addition, to estimate the free lysine in the samples, the baseline level of water-extractable amino groups was halved during calculations performed on the raw data, as a free lysine moeity contains 2 amino groups.

was (a) not being measured by the method in use; (b) had fallen off the pellet immediately after being sprayed on; (c) was not present in the lysine solution to begin with; (d) had reacted *via* the Maillard reaction prior to analysis; or a combination of these. Whether this was due to methodology (option a) or due to an actual decrease in reactive/bioavailable lysine (options b-d) can be determined by comparing measure lysine contents with bird performance. As Diets 1 and 8 have similar theoretical free lysine contents, a methodology error would indicate that birds should perform equally on both diets. However, if the method was accurately measuring lysine, birds should perform better on Diet 1. This comparison is discussed in section 5.6.1.

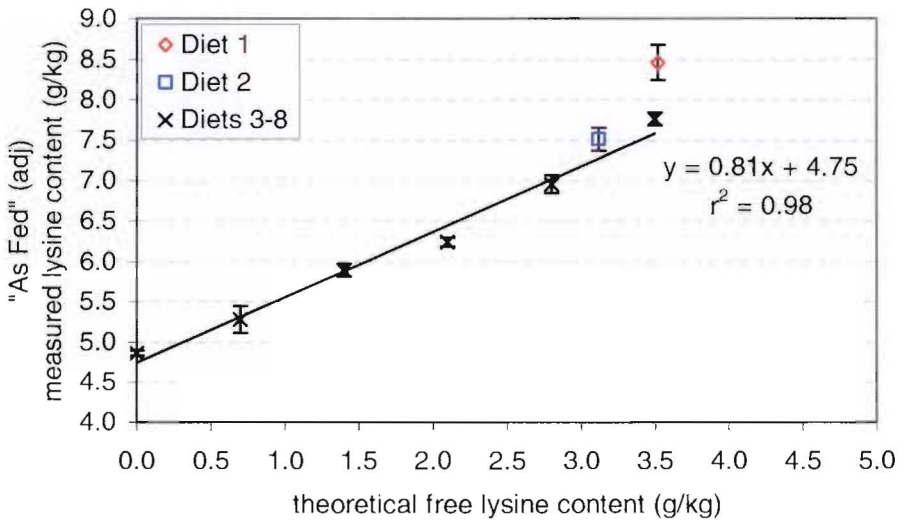

Figure 5.4-b: Adjusted measured lysine results compared with adjusted lysine addition values for Diets 1-8. The calibration is shown for Diets 3-8 only. The standard feeds for New Plymouth (Diet 1) and Christchurch (Diet 2) are included for comparison. Error bars represent standard error of 10 replicates.

It is also improbable that the lysine fell off immediately after being sprayed on to the pellet (option b), as samples were taken immediately after the spraying process had occurred, such that any lysine that had fallen off should have still been retained within the sample. Also, if the lysine had fallen off, it would have been expected that this would have occurred

to an increased degree over the time scale of the experiment, such that the "Final" values were lower than the "Initial" values, which was not seen (Figure 5.4-a).[a]

It is doubtful that option (c) occurred as a result of lysine degradation, given the stability of free lysine, as evidenced by autoclaving trials in chapter 4. However, it is possible that the lysine source was impure, or that errors occurred in the formulation of the lysine solution prior to application.

This means the difference between theoretical and measured lysine contents was due to (a) methodology; (c) the lysine solution being made up incorrectly or (d) lysine degradation due to Maillard reaction, occurring to an equal degree to all samples with lysine added post-pelleting. This is discussed further in section 5.6.1. Samples taken from a follow-up growth trial (Appendix III) were also analysed for lysine content as part of this research. Lysine contents were consistent with those shown in Figure 5.4-b.

5.4.3 Lysine contents of diets remaining in feed troughs

Samples of distributed but uneaten feed were collected, pooled for each feed type, and sieved to separate fine powder from the coarser material and pellets (Figure 5.2-a). The percentage of fine powder in each pooled sample ranged from 17% to 27% (w/w fines/total uneaten sample), with an average of 23% (standard error 1.2%). While no complete uneaten samples (*i.e.* the entire uneaten contents of a feed trough at the end of the trial) were taken, this was likely to be a valid reflection of the fines content in the troughs, due to thorough mixing prior to sample collection. Hence, 23% was used as the percentage of fines per trough for subsequent calculations.

The "Coarse" and "Fines" samples had four extractions performed on each diet, and lysine assays were carried out in triplicate on each extraction. The results are shown in Figure

[a] This was a separate issue to the formation of high lysine levels in the "Fines" samples, as discussed in section 5.4.3, where the loss of lysine, from the pellet to the fines, led to a reduction in the "As Fed" diet values, not to the theoretical values.

5.4-c, in conjunction with prior results for the lysine contents of each diet ("As Fed"), with all data adjusted for moisture content as outlined in section 5.4.2.

From this figure, it appears that the coarse residues contain approximately 5% more lysine than the respective diets as fed, but as the lysine content of the coarse residue of Diet 3 (no lysine added) was greater than that for Diet 3 as fed, this was probably just a difference in protein extraction levels. However, it was obvious that the lysine content of the fine residue from Diets 4-8 were significantly higher than for the coarse residue of the equivalent diets. This result was unsurprising, as one would predict that lysine sprayed onto the outside of the pellet would be far more susceptible to breaking off with other pellet material to become part of the fines. This increase in lysine in the fines was not seen in Diets 1 and 2, where the lysine was incorporated into the meal prior to processing.

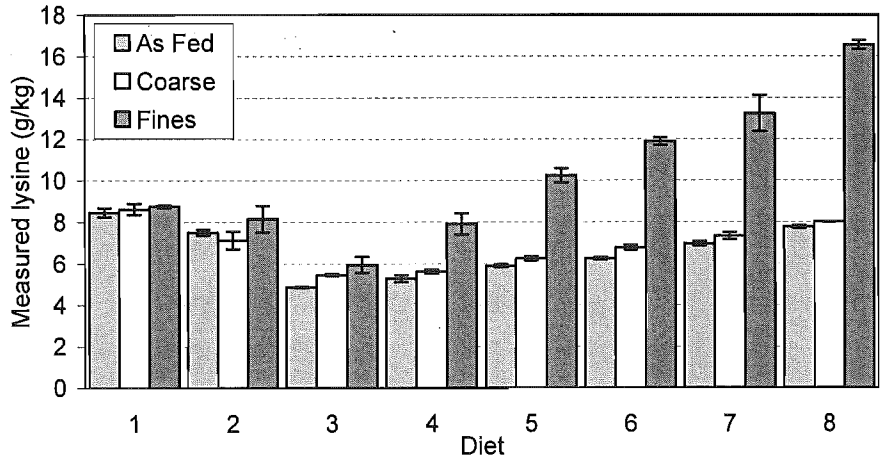

Figure 5.4-c: Comparison of lysine contents of Diets 1-8 as fed, coarse feed left in trough (coarse) and fine feed left in trough (fines). Error bars represent the standard error of 10 replicates for the As Fed samples, and 4 replicates for the Coarse and Fines samples.

On comparison of the results from the fine residue of Diets 3-8 with the amount of (adjusted) added lysine, a high correlation was seen (Figure 5.4-d). However, the slope of this correlation was 2.89. This was 3.55 times greater than the slope seen for the

comparative "As Fed" values, indicating that there was 355% more lysine in the fine powder than in the original pellets as fed to the birds. This large difference in the lysine content of the "Fines" relative to the "As Fed" data indicates that lysine which had been sprayed onto the pellet had subsequently come off, probably as a result of normal pellet break-down.

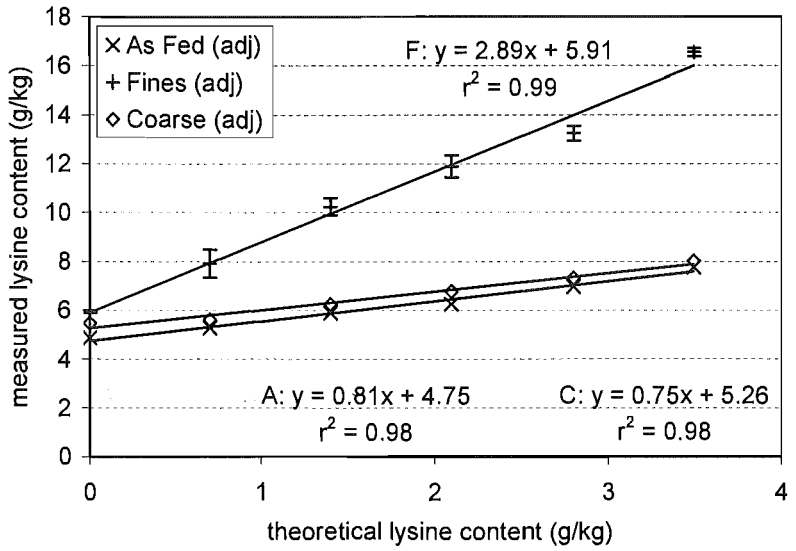

Figure 5.4-d: Theoretical lysine content of Diets 3-8, compared with the "As Fed" and "Fines" and "Coarse" adjusted lysine contents. Calibration equations shown for A: "As Fed"; C: "Coarse"; F: "Fines". Error bars represent standard error of 4 replicates; error not shown for "As Fed" or "Coarse" as obscured by data points (error of 0.1 g Lys/kg for both series).

5.5 CALCULATION OF THE LEVEL OF FREE LYSINE EATEN PER BIRD ON EACH DIET

In order to determine how well the reactive lysine levels measured *via* the OPA method compared to bird performance, the average amount of free lysine eaten per bird per diet

had to be calculated. In conjunction, the average amount of free lysine eaten per bird per diet was required to compare the effectiveness of adding lysine pre *versus* post-pelleting.

To calculate the total amount of free lysine eaten during the trial by each bird, the base extracted level of reactive amino groups had to be determined, so that the free lysine in each sample could be ascertained. This base extracted amino group level was determined for the "As Fed" diet from the intercept of the calibration shown in Figure 5.4-d (A), giving a base extraction level of 4.75 grams of lysine equivalents per kilogram of feed. Similarly, "Coarse" and "Fines" samples had a base extraction level of 5.26 and 5.91 g lysine/kg feed respectively, determined from the calibration intercepts shown in Figure 5.4-d (C and F). Subtracting this value from each recorded lysine measurement gave an estimate of the free lysine in each sample. For example, to estimate the amount of free lysine in Diet 1, the following calculation was performed:

Free lysine = "As Fed" (adj) – base extracted amino group level

Free lysine = 8.46 – 4.75

Free lysine = 3.71 g Lys/kg feed

In addition to determining the amount of free lysine in each sample, the average amount of lysine, per bird per diet, which remained uneaten in the feed troughs at the end of the trial was calculated. For this determination, 23% of the uneaten portion was assumed to be fine powder, as stated in section 5.4.3. A total amount of 9000 g of feed was distributed to each cage of birds over the course of the experiment, and on average 1261 g of this remained uneaten at the end of the trial.

Using the free lysine contents in each sample for each diet, as shown in Table 5.5-a, the total amount of lysine available for the chicken to eat was calculated, along with the total amount of lysine not eaten by the chicken. Hence, the average amount of free lysine eaten per bird per diet could be calculated (for details of this calculation, see Appendix I). Of the total amount of free lysine added to each kilogram of pellets, on average 10% of this (Diets 4-8, standard error 1%), over what would be expected, remained in the uneaten fine

powder (compared to no extra for Diets 1 and 2, as fed). This indicates that an additional 10% of the lysine, added as a liquid, was not eaten by the chickens in the trial. This 10% was factored into the results for Diets 4-8, as fed, by reducing the values above basal level (Diet 3), obtaining the "As Eaten" lysine contents visualised in Figure 5.5-a.

Diet	Theoretical [a] (g Lys/kg feed)	As Fed (adj) (g Lys/kg feed)		Coarse (adj) (g Lys/kg feed)		Fines (adj) (g Lys/kg feed)	
	Free	*Total* [b]	*Free* [c]	*Total* [b]	*Free* [c]	*Total* [b]	*Free* [c]
1	3.52	8.46	3.71	8.63	3.37	8.76	2.85
2	3.12	7.51	2.76	7.13	1.87	8.16	2.25
3	0	4.86	0.12	5.46	0.21	5.94	0.03
4	0.70	5.28	0.53	5.62	0.36	7.91	2.00
5	1.40	5.89	1.15	6.24	0.98	10.24	4.33
6	2.10	6.24	1.49	6.77	1.51	11.89	5.98
7	2.80	6.96	2.21	7.32	2.07	13.24	7.33
8	3.50	7.77	3.02	8.01	2.75	16.55	10.64

[a] Theoretical amount of added lysine. Values from Table 5.4-a.

[b] Total amount of water extractable lysine as measured by the OPA method. Values calculated as in Table 5.4-a.

[c] Calculated amount of free lysine in each sample, using the method described in the text.

Table 5.5-a: Calculated values for total and free lysine content of Diets 1-8 for sample sets "As Fed", "Coarse" and "Fines", compared with the theoretical value of added free lysine.

Figure 5.5-a clearly shows that while birds on Diets 1-3 ate feed with free lysine contents close to that theoretically added, birds on Diets 4-8 ate significantly less free lysine than predicted from the theoretical values. This highlights the importance of *in vitro* lysine testing in conjunction with growth trials, as it may have otherwise been assumed that birds on diets 4-8 were underperforming in relation to the theoretical amount of free lysine that they were consuming. The fact that Diet 2 was slightly lower than theoretically predicted was most likely due to the different formulation of this diet leading to a different base extraction level of amino groups than predicted by Diet 3.

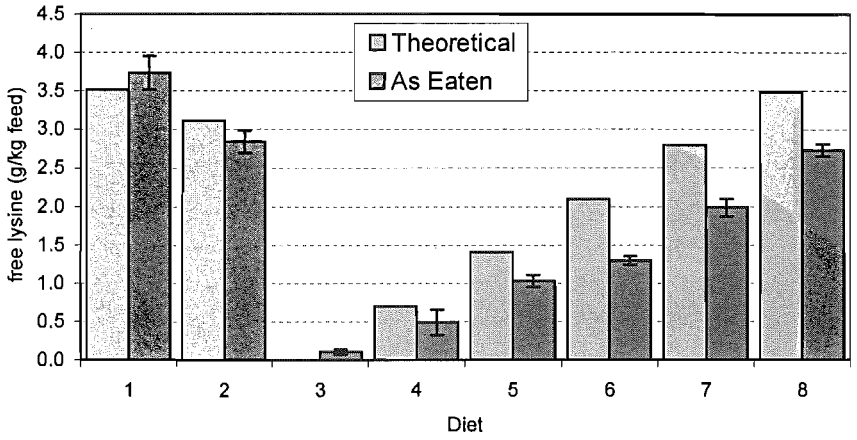

Figure 5.5-a: Comparison of free lysine contents of Diets 1-8, as eaten, with the theoretical amount of free lysine added to each diet. Error bars represent standard error of 5 replicates.

5.6 BIRD PERFORMANCE IN THE GROWTH TRIAL

Bird performance was evaluated in this trial using the FCR, calculated for the chicks aged day 8-14 and day 15-21. Typical results indicate birds utilise feed more efficiently at younger ages, and therefore FCR values increase with age,[3] as a lower FCR value represents a better conversion from feed to weight gain. In the results for this growth trial, which are shown in Figure 5.6-a, FCR did increase for all diets from week A to week B.

These data clearly show the importance of lysine in the diet of chickens, as the birds fed Diet 3, with no added lysine, showed poorer feed conversions than birds on all other diets, for both trial weeks. These data also show that lysine was the limiting growth factor of Diet 3. It is also obvious from Figure 5.6-a that birds on the standard New Plymouth F1 finisher diet (Diet 1) outperformed all others in week A of the trial, while birds on the standard Christchurch F1 finisher diet (Diet 2) performed similarly to birds on Diets 4 and 5 in week A. In week B, however, birds on Diets 1 and 4-8, all performed to a very similar degree.

As Diet 2 (Christchurch standard F1) has a different basic ingredient make-up from the remaining 7 diets, it would be expected that bird performance would differ from expected when correlated with water-extractable lysine levels. Hence, for the purpose of correlating FCR with measured lysine contents, the Diet 2 results were discarded.

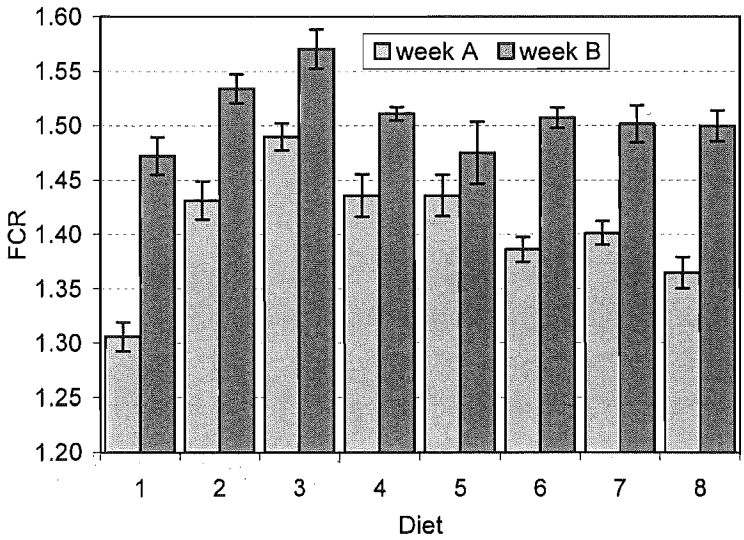

Figure 5.6-a: Bird performance, as measured by FCR values, on each of the diets for week A and B. Error bars represent standard error of 8 replicate cages for each diet.

5.6.1 Comparison of measured lysine contents with FCR values from the growth trial.

Bird performance was markedly different between for week A and week B, as evidenced by Figure 5.6-a, therefore the FCR values for each week were compared to lysine contents separately, and then overall results were investigated.

Comparison between FCR values during Week A and measured lysine contents

In Figure 5.6-b, week A FCR values are plotted against the measured lysine contents for Diets 1, 3-8. The measured lysine content correlates with the FCR very strongly, with an

r^2 = 0.90. This shows clearly that lysine was the limiting amino acid for these chicks in day 8-14 of life.

In week A, it can be seen that birds on Diet 1 (standard New Plymouth diet, circled in Figure 5.6-b) performed significantly better than birds on Diets 3-8, and the FCR of birds on Diet 1 fitted well with the growth response curve for lysine intake (Figure 5.6-b). As discussed in 5.4.2, this indicates the methodology for testing lysine was good, as the 19% reduction between theoretical and measured lysine content of Diets 4-8, seen in Figure 5.4-b, equated with bioavailable lysine contents, as determined by this growth trial (week A). Thus, from the discussion in section 5.4.2, this leaves options (c) the free lysine was not present at application due to an impure lysine source or because of preparation error or (d) Maillard reaction had occurred in all samples with lysine added post-pelleting, such that an consistent percentage loss of lysine was seen.

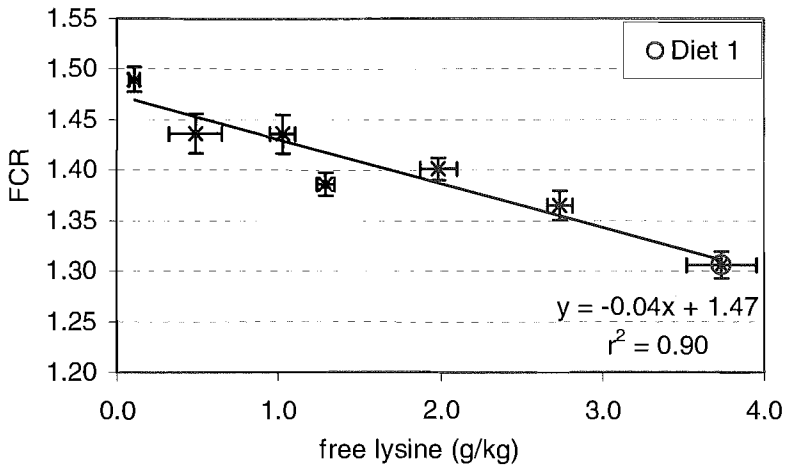

Figure 5.6-b: The correlation between measured lysine content and week A FCR values, for Diets 1, 3-8. Measured lysine is shown as the "As Eaten" free lysine values, from Figure 5.5-a. Error bars represent standard error – FCR: 8 replicate cages; measured lysine: 10 replicate extractions.

As discussed in chapter 4, FCR values are calculated from feed intake and bird growth. Therefore, a lower FCR value could reflect increased bird growth or decreased feed intake. In Figure 5.6-c the influence of dietary free lysine content on feed intake is examined.

From this, it is apparent that per gram increase in free lysine content of the feed, feed intake increased by 7 grams. Therefore, the lower FCR values that resulted from increased levels of free lysine in the diets, as seen in Figure 5.6-b, reflected greatly improved growth relative to feed intake.

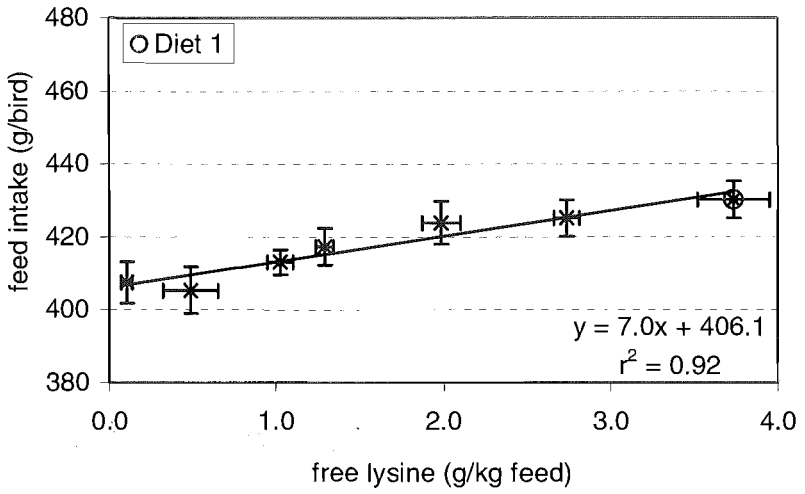

Figure 5.6-c: The influence of the free lysine content ("As Eaten") of Diets 1, 3-8 on feed intake during week A. Error bars represent standard error – feed intake: 8 replicate cages; measured lysine: 10 replicate extractions.

Previous research has shown that feed intake is depressed as a general response to an imbalance in amino acids.[4] The feed intakes for birds on each treatment, shown in Figure 5.6-c, were consistent with this finding, as feed intake increased as the lysine imbalance was reduced.

In order to ascertain the amount of growth supported by free lysine in week A, the amount of free lysine consumed per bird per diet was calculated. In Figure 5.6-d this is correlated with bird growth, and shows that for every additional gram of free lysine consumed, an additional 30 grams of bird growth was supported. As this was much greater than the increase in feed intake resulting from higher lysine contents, the decreasing FCR values seen in Figure 5.6-b are explained. It is also interesting to note the very high correlation between bird growth and free lysine consumed (r^2=0.96), again highlighting the importance of lysine as the growth limiting factor in the diets of these chickens.

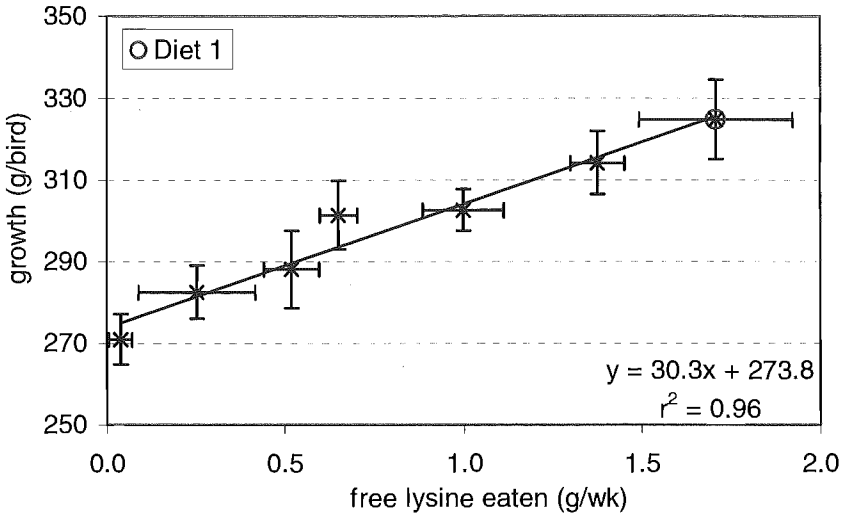

Figure 5.6-d: The influence of the amount of free lysine eaten, for birds on Diets 1, 3-8, on bird growth during week A. Error bars represent standard error – growth: 8 replicate cages; measured lysine: 10 replicate extractions.

Comparison between Week B FCR values and measured lysine contents

The data for week B of the trial (chickens age day 15-21) were very different to those of week A (Figure 5.6-e). Under the liquid lysine post processing addition conditions used, any lysine addition above the first addition level (0.875 kg lysine-HCl/tonne, Diet 4) led to no further significant increase in bird performance.[a]

Given that storage history may be a factor in bird performance, it was noted that recent research has indicated that staling can occur in feeds 2-4 weeks after processing, and that this can influence bird performance through decreasing feed intake.[5] However, for Diet 1, data for feed intake approximated standard Tegel chicken farm feed intakes,[3] therefore staling was not a significant factor in this trial.

It is possible that, in week B, the OPA method was measuring lysine as reactive, but the free lysine had altered, *via* Maillard reaction, during the course of the trial so as to be no

[a] This was a previously unknown result for Tegel.

longer a bioavailable source of lysine for the chicken. However, the fact that all lysine addition levels produced an equivalent increase makes this unlikely, as it indicates that all but 0.7 g Lys/kg reacted to become unavailable, irrespective of lysine addition level. This would be a more plausible explanation if no lysine addition level produced a growth response, or if the growth response was still proportional to lysine addition, as these would indicate either all lysine had reacted to become unavailable, or that a consistent percentage had.

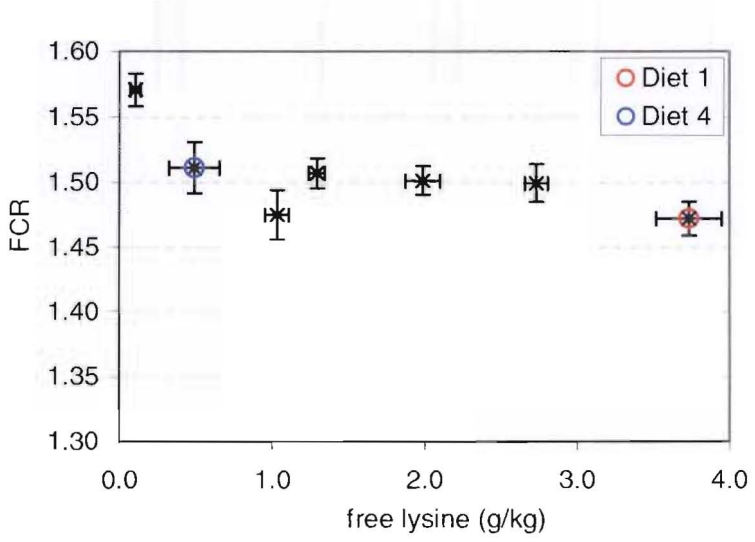

Figure 5.6-e: The correlation between measured lysine content and week B FCR values, for Diets 1, 3-8. Measured lysine is shown as the "As Eaten" free lysine values, from Figure 5.5-a. Error bars represent standard error – FCR: 8 replicate cages; measured lysine: 10 replicate extractions.

Comparison between overall FCR values and measured lysine contents

Overall FCR values were calculated from total growth and total feed intake data over the two week period of the trial. From these FCR values, the relative effect of week A and week B bird performance can be assessed. As birds both ate and grew more during week B, performance data from this week has a greater influence on overall FCR values, as plotted in Figure 5.6-f.

Overall FCR values were not significantly different ($P>0.05$) for Diets 4-8. However, Diets 3 and 4, and Diets 1 and 4 were significantly different ($P<0.05$). The majority of the disparity in overall FCR values between birds fed Diets 1 and 4 originated from differences in growth in week A. It would be interesting to see if this was also true if lysine had been added prior to pelleting in all diets. Assuming no difference did exist between pre and post-pelleting lysine addition, these results indicate that the most economical growth could be achieved by feeding at the 100% level for birds aged day 8-14, and at the 20% addition level for birds aged day 15-21, resulting in a decrease of 50% of free lysine used over the two week period.

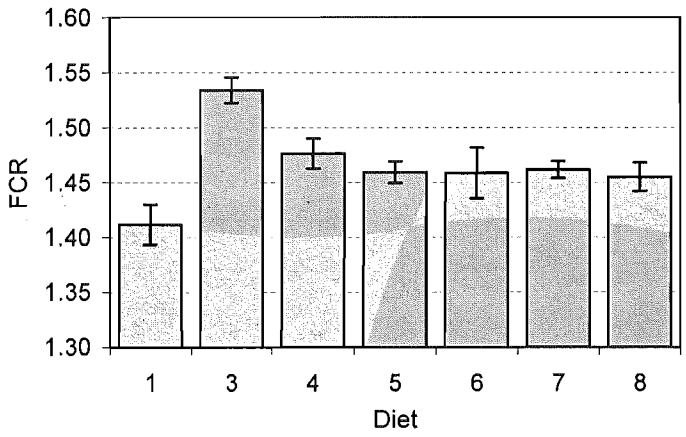

Figure 5.6-f: The overall FCR values, for Diets 1, 3-8. Error bars represent standard error of 8 replicate cages.

While any effects of this decrease over the lifetime of the bird needs to be examined in detail, this was an extremely significant result. Not only could large savings be made due to the decreased need for lysine, but the potential for improved bird performance exists if a new growth limiting factor can be found for this period. It would be necessary to determine whether this is replicated for similar levels of lysine addition pre-pelleting prior to further investigation. In future research, it would be interesting to determine whether the same result would have occurred if the Christchurch feed had been used as the lysine free basal diet.

Comparison between measured lysine and added lysine for Diets 1 and 2

Using data presented in Table 5.5-a, it can be seen that Diet 1 (New Plymouth standard F1) contains 3.71 kg/tonne of lysine, or 4.64 kg/tonne of lysine-HCl. This compares well with the theoretical lysine-HCl addition value of 4.40 kg/tonne. The closeness of these values would indicate that no Maillard reaction of significance has occurred with the free lysine added to feed during the processing procedure.

Similarly, Diet 2 (Christchurch standard F1) contains 2.8 kg/tonne of lysine, or 3.5 kg/tonne of lysine-HCl more than the New Plymouth basal level. As the theoretical addition level for Diet 2 was 3.9 kg/tonne, this would indicate that the (water extractable) basal level for the Christchurch feed was approximately 0.3 kg/tonne lower that for the New Plymouth feed.

5.7 CONCLUSIONS

There was a strong correlation (r^2 = 0.90) between bird performance in week A (8-14 days of age) on the trial diets, and the measured lysine content. Increased lysine addition levels correlated well with both increased feed intake and bird growth. Therefore, lysine was the limiting factor in the bird's diet in week A of the feed trial. In addition, the OPA method for lysine testing was valid under the conditions used.

The value of lysine added as lysine-HCl, predicted by chemical analysis, of the New Plymouth feed was 4.6 kg/tonne, which was very close to the theoretical value of 4.4 kg/tonne. From this result, it appears no significant loss of free lysine occurs during the pelleting process. Any Maillard reaction that does occur uses up only a small fraction of the free lysine added, but this may result in bioactive compounds, which only need to be present in very small quantities.

Diets 4-8 in the first growth trial were subject to a 19% difference between theoretical and measured lysine contents. As this was not seen to such a degree in the follow-up trial (Appendix III), and as the growth trial showed good equivalence, in week A, with measured lysine, it was determined this was most likely due to an impure lysine source or errors in lysine application.

It would appear that spraying lysine on to pellets post-processing was not an effective or economically viable method of delivering lysine to the chickens. Approximately 10% of the lysine added as a liquid could be found in the fine powder left in the feed troughs at the end of the trial. This would indicate that any ingredient added to the outside of a pellet should have a 10% loss factored in to its application, or that the application method must be improved.

These differences between theoretical and measured, and "As Eaten" lysine contents illustrate the importance of *in vitro* lysine testing in growth trials such as this, to ensure that theoretical levels of lysine in the feed equate to what is actually being eaten by the bird. Otherwise, inaccurate assumptions could be made about the growth results seen.

In the week B (15-21 days of age) on trial feed, no increase in bird performance can be seen with increased lysine addition levels above the first level (Diet 4 – 0.875 kg/tonne of lysine-HCl added). Therefore, only 20% of the current lysine addition level was necessary to deliver equivalent bird performance in the third week of bird life. Over 2 weeks, this would equate to a 50% saving in the amount of free lysine addition required. In conjunction, some other factor was limiting growth in this week of life, and if this limiting factor could be determined, bird performance could be increased. Although incidental to the hypothesis under study, this was a very significant result.

5.8 REFERENCES

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SUMMARY AND CONCLUSIONS

CHAPTER 6

This thesis investigated the occurrence of the Maillard reaction under feed processing conditions, and the nutritional significance of this reaction during the pelleting of chicken feed.

To investigate the lysine modification and protein crosslinking that could occur during feed processing or storage, model systems containing RNase A and either a pure or feed type carbohydrate were studied at a variety of temperatures. These showed that apparent lysine loss can occur at room temperature immediately upon mixing a reducing carbohydrate with a protein. This was most pronounced after mixing of RNase A with cyclotene, glucose, malt extract or molasses. Mixing with xylose or sucrose produced a much smaller reduction in the lysine content of RNase A, whereas starch or dextrin produced no significant reduction. The standard error of this initial drop in lysine content was much greater when molasses, malt extract or starch were in the system, due to impurities present.

On incubation with cyclotene at 37°C, RNase A dimer formation was visible at 9 days, trimer formation at 26 days and tetramer formation at 29 days. The amino group content of the incubation samples decreased until day 15, at which point no further loss of lysine was seen. It was determined that this was due to protein fragmentation, with concurrent formation of N-terminal groups, masking reactive lysine loss over the course of the incubation. This was supported by results showing arginine was not involved in the crosslinking of RNase A *via* cyclotene to any significant degree.

Amino group contents of 37°C incubation samples containing RNase A with a pure carbohydrate were the least complex, indicating that protein fragmentation and Maillard

reaction were occurring at near-linear rates. With feed-type carbohydrates, the amino group profiles of RNase A reaction became more complex. This was particularly pronounced after incubation of RNase A with molasses, which increased the rate of protein fragmentation dramatically in some incubation samples.

Changing the temperature of incubation from 37°C to 70°C significantly increased the rate of crosslinking reaction in some samples. This was most pronounced when cyclotene or xylose were present. A high degree of protein aggregation and fragmentation was observed after incubation of RNase A at 70°C for 12 hours, with or without carbohydrate present.

Extending from results gained in model systems using RNase A, the reaction of flour proteins with various carbohydrates, with and without the addition of lysine were investigated. To quantify the lysine content of these non-aqueous soluble flour proteins, the OPA method was adapted for analysis of the total protein extracts of barley flours. Good concurrence between the mOPA method and the ninhydrin method for the lysine contents and the Maillard reacted lysine contents of 36 barley flours.

Utilising the ninhydrin and the mOPA methods to test the lysine content of 4 barley cultivars grown at 9 different sites, good equivalence was found in the measured levels of Maillard reacted lysine. The degree of Maillard reacted lysine was dependent on site of growth, with barley grown at Balfour averaging 25% blocked lysine. This highlights the importance of testing feed ingredients for lysine loss if the nutritional quality of the final product is important.

After autoclaving flour with a variety of carbohydrates, a high degree of crosslinking was seen with xylose, malt extract and molasses. Flour samples autoclaved with starch or dextrin produced crosslinking profiles similar to that seen for flour autoclaved without added carbohydrate. The addition of as little as 1% lysine or 5% lysine increased reaction in the flour systems, and this was particularly pronounced when both were added to flour. These model systems clearly showed the potential for nutritional damage during processing. Furthermore, damage occurred to flour proteins that had not had a carbohydrate source added prior to processing.

Lysine analysis of samples taken from a commercial feed mill as mash, ex-conditioner and pellets indicated losses of at least 18% could occur during pelleting. The occurrence of protein fragmentation due to the shear forces during pelleting masked further losses. Lysine analysis of feeds with selected amino acids (lysine, methionine and/or threonine) not added to the mash indicated loss was highly dependent on processing conditions.

As the pelleting procedure has the potential to cause lysine to become nutritionally unavailable, methods of preventing this nutritional damage were investigated. Lysine protection *via* TGase catalysed crosslink formation was studied, but did not appear to protect lysine in systems modelling the pelleting process. It was determined that the novel method of spraying lysine solution onto the pelleted feed was an economical and technically feasible method for preventing Maillard reaction damage of the lysine during pelleting.

A growth trial investigated the utility of post-pelleting lysine application. Lysine analysis of diets at the start and end of the trial indicated that no lysine was lost as a result of storage. However, lysine contents of diets with lysine added post-pelleting were not as high as would be expected from theoretical lysine addition levels. Analysis suggested this was due to an impure lysine source or inaccurate formulation of lysine solutions.

Analysis of the fines remaining uneaten at the end of the trial showed a high content of lysine for diets with lysine added post-pelleting. At the end of the trial, 10% of the free lysine initially sprayed on to the pellet was present in the uneaten fines. Therefore, this loss needs to be taken into account for any feed ingredient sprayed on post-processing.

In the first week of the growth trial (8-14 days post-hatching) very good correlation was seen between the amount of lysine eaten per bird, calculated from lysine contents measured using the OPA method, and bird growth. This showed that lysine was growth limiting at this point in the birds' lives. In addition, as the lysine content of the diet increased, a linear increase in feed intake was observed. This increase in feed intake may reflect the increasing energy requirements of the birds due to the higher level of growth supported by the increased lysine content of the diet.

Bird performance in the second week of the growth trial (15-21 days post-hatching) was very different to that of the first week. Lysine was no longer growth limiting above the first addition level, as no significant improvement seen in bird performance at higher addition levels. Over both weeks, the standard diet gave the best performance. It is possible that equivalent performance could have been achieved, however, with a 50% saving in the overall level of free lysine added to the diet over this period.

On comparison of the measured lysine content of the standard diet with the theoretical amount of lysine added to the diet, no lysine loss occurred as a result of processing. This was supported by bird growth data in week 1 of the trial, where measured lysine correlated well with bird growth.

Overall, lysine loss during chicken feed processing is not significant under the pelleting conditions used to process the feed for the growth trial. However, results indicate variations between runs could cause significant loss to occur. Given that up to 25% blockage of lysine in unprocessed barley flour samples was observed, sourcing feed ingredients that have consistent and low levels of Maillard reaction damage to the lysine contained in them may be as important as maintaining ideal processing conditions.

EXPERIMENTAL

CHAPTER 7

7.1 GENERAL METHODS

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company Ltd., Aldrich Chemicals or BDH Laboratory Supplies, and were generally of analytical grade. Solvents were purchased from BDH and were of analytical grade.

Ribonuclease A (RNase A) was Type XII-A (from bovine pancreas) from Sigma.

pH was measured using an EDT Instruments BA 350 series 3 pH meter fitted with an EDT Instruments E8030 electrode, calibrated against standard buffers at pH 4.0, pH 7.0 and 10.0. Alternatively, pH was measured on a Metrohm 718 STAT Titrino pH stat, fitted with a Metrohm micro pH probe (6.0204.100), calibrated against the same standard buffers. The pH stat was run by a personal computer running Metrohm Titrino software.

Dialysis was undertaken using Pierce Slide-A-Lyzer mini dialysis units (MWCO 7 kDa) or Sigma dialysis cellulose membrane tubing (MWCO 12 kDa, prepared as per instructions on packaging).

All SDS-PAGE of RNase A, unless otherwise noted, was carried out using Gradipore precast 8-16% acrylamide gradient iGels (NSW, Australia), a Bio-Rad Mini-PROTEAN® III kit and a Bio-Rad 300, or a Bio-Rad 1000, Power Pack. SDS-PAGE of whole flour protein extracts were carried out using hand-made gels, a Bio-Rad PROTEAN® II xi Cell kit and a

Bio-Rad 300 Power Pack. Acrylamide monomer solution contained 30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide. SDS-PAGE gel images were captured using a Nikon D1x digital camera fitted with a Nikon 60 mm macro lens.

Ultraviolet (UV) spectroscopy was carried out on a Hewlett Packard 8452A Diode Array Spectrophotometer interfaced with a personal computer running Hewlett Packard 8452A UV-visible operating software.

All cuvettes, unless otherwise specified, were 1 cm path length plastic cuvettes from Sarstedt Inc., supplied by Global Science.

Fluorescent spectroscopy was carried out on a Cary Eclipse fluorometer interfaced with a personal computer running Cary 1.0 operating software, at Canterbury Health Laboratories.

Microtitre plates used for fluorometric measurements were Nunc untreated black 96 microwell plates.

Centrifugation of 1.5 mL Eppendorf tubes was performed in Eppendorf 5403 Centrifuge or an Eppendorf Mini-Spin Plus centrifuge (12 x 1.5 mL fixed-angle rotor, max *g*-force, 14,000 x *g*). 10 mL tubes were centrifuged in a Heraeus Christ Labofuge.

Freeze drying was performed in an Edwards "Speedivac" Model 30P2 Centrifugal Freeze Dryer.

Samples were milled to 1 mm by a Janke and Kunkel Culatti MFC Micro Mill, using a 1 mm mesh. Samples were milled to 0.5 mm using a Tecator 'Cyclotec' Model 1093 Sample Mill, using a 0.5 mm mesh, at Crop and Food Research.

Autoclaving was carried out in a Mercer PLC Type 65 Automatic Adjustable Steam Sterilizer, under a normal wet heat sterilization of 20 minutes at 121°C with pressure at 10.3 kPa.

Broiler F1 feed meals and pellets were provided by Tegel Foods Ltd.

Growth trials were undertaken at the Monogastric Research Unit at Massey University, Palmerston North.

7.2 EXPERIMENTAL FOR WORK DESCRIBED IN CHAPTER 2 – METHOD DEVELOPMENT

7.2.1 Protein concentration determination

Standard Bradford method for protein concentration determination

RNase concentrations were initially estimated using the micro version of the Bio-Rad Protein assay,[1] which is based on the Bradford method.[2] RNase A samples were diluted to give protein concentrations of approximately 60 μg/mL. 80 μL of each diluted sample was added to a cuvette containing 720 μL of distilled water, and then to initiate the assay 200 μL of Bio-Rad Bradford solution was added to each cuvette, which was mixed and incubated at room temperature for 6 minutes. The absorbance of each sample was then read at 595 nm against a distilled water blank. For calibration purposes, an RNase A standard curve was used. For this, protein concentrations of 20 μg to 110 μg, prepared in the same manner as the samples, were assayed in triplicate.

The modified Bradford method for RNase A concentration determination

The standard method was modified, as suggested by Duhamel *et al.*,[3] such that the 80 μL diluted sample was added to a cuvette containing 720 μL of 41.7 μg/mL SDS solution (*i.e.*

30 µg SDS), instead of 720 µL of distilled water. This was then incubated at room temperature for at least 15 minutes prior to standard Bradford analysis.

Determination of sub-threshold level of SDS for the Bradford assay

To determine the sub-threshold level of SDS, *i.e.* where SDS by itself showed no absorbance for the Bradford method as utilised, SDS solutions were prepared in concentrations ranging from 0 mg/L to 100 mg/L. 800 µL of each concentration was placed in a cuvette, and 200 µL of Bradford solution was added to each cuvette and mixed. The samples were incubated for 6 minutes at room temperature, and the absorbance of each solution read at 595 nm against a solution blank without SDS. The highest concentration of SDS that did not increase absorbance above that of the blank was taken as the sub threshold level used for all subsequent RNase A protein concentration readings. This sub-threshold concentration was determined to be 30 µg per sample; *i.e.* 30 µg/mL for the final cuvette volume (800 µL SDS solution + 200 µL Bradford solution).

7.2.2 The OPA method

The OPA (*o*-phthaldialdehyde) method for testing lysine availability in water-soluble proteins was from Bertrand-Harb *et al.*[4] The OPA solution was made daily by combining the following:

25 mL sodium borate (0.1 M)

2.5 mL SDS (20% (w/v))

40 mg OPA dissolved in 1 mL methanol

100 µL 2-mercaptoethanol

The final volume was adjusted to 50 mL with distilled water.

To assay lysine availability, a 50 µL aliquot of sample containing approximately 2 g/L protein was added to 1.0 mL of OPA reagent in a cuvette. The solution was mixed using a pipette tip and incubated for two minutes at room temperature. The absorbance was then read against a blank of distilled water at 340 nm. All samples were assayed in triplicate. Calibration curves were established using lysine and RNase A. In the cases where lysine was being used as a standard for protein-bound lysine content, the slope of the resultant calibration curve was halved to take into account the two free amino groups of free lysine.

7.2.3 The modified OPA (mOPA) method

A modified version of the *o*-phthaldialdehyde (OPA) method was developed to estimate lysine availability in barley proteins. OPA was freshly prepared by combining the following:

25 mL bicine (1.6% (w/v), pH 9.4)

2.5 mL SDS (20% (w/v))

40 mg of OPA dissolved in 1 mL methanol

100 µL 2-mercaptoethanol

The final volume was adjusted to 50 mL with 1-propanol.

Lysine availability was assayed, and calibration curves were determined as for the standard method.

7.2.4 The ninhydrin method

The ninhydrin method for testing lysine content of flour proteins was based on the method developed by Friedman *et al.*[5]

Reagents:

Lithium acetate buffer (4 M):

168 g lithium hydroxide

400 mL deionised water

300 mL glacial acetic acid

The pH of the solution was adjusted to 5.2 ± 0.05 with acetic acid or lithium hydroxide.

The solution was made up to 1 L with water and stored in a plastic bottle at room temperature.

Ninhydrin Solution:

2.0 g ninhydrin

0.3 g hydrindantin

75 mL dimethyl sulfoxide (DMSO) (100%)

This solution was mixed, and added to:

25 mL lithium acetate buffer (4 M)

The solution could be stored for 1-2 weeks at 4°C, under nitrogen.

Lysine analysis

Samples containing approximately 4.5-8.5 mg of flour (in duplicate or triplicate) were weighed into 10 mL screw-lid plastic centrifuge tubes.

To each tube, 2.0 mL distilled water along with 2.0 mL ninhydrin solution were added. A reagent blank was also set up, containing the water and ninhydrin solution but no flour. The lids were screwed loosely onto the tubes and each sample was vortexed briefly. The

tubes were then placed in a boiling water bath for 15 minutes. During this incubation period, each tube was vortexed briefly, at 5 minute intervals. At the end of this incubation period, the samples were cooled in ice water and 6.0 mL of a 50% ethanol-water solution was added to each tube. The tubes were vortexed and any ninhydrin-negative, insoluble particles were separated by centrifugation. The supernatant was decanted for analysis. The absorbance of each sample at 570 nm was then measured against a reagent blank. If necessary, the reaction mixture was diluted with additional 50% ethanol to place the absorbance in the linear range of the spectrophotometer.

7.2.5 Total protein extraction from barley flours

Standard method

Hordein proteins were extracted from barley flour according to the method of Marchylo and Kruger.[6] 0.05-0.1 g of the flour was placed in an Eppendorf tube with 400 μL of the extracting solution (50% (v/v) 1-propanol containing 1% (w/v) dithiolthreitol (DTT)). This mixture was then vortexed and placed in a 60°C water bath for 30 minutes, with vortexing at 10 minute intervals during this period. Extracts were then centrifuged at 4300 *g* for 5 minutes and the supernatants collected and diluted to an appropriate concentration for lysine analysis. Lysine analysis *via* the mOPA method was carried out on these supernatants on the same day.

7.2.6 Comparison of lysine contents of 36 barley flours as measured by the mOPA method and the ninhydrin method

36 barley flours – 4 cultivars grown at 9 sites - were provided by Crop and Food Research, Lincoln.

Modified extraction method

This method was carried out in the same manner as described in section 7.2.5, except that extraction was performed at room temperature.[7] The samples were placed in a custom

made multi-Eppendorf holder, such that multiple samples could be constantly vortexed. The samples were then vortexed overnight, and treated as in the standard method.

To analyse the modified extraction method, 5 flour samples were randomly selected, and extracted by both methods. The extract was then tested for lysine content in triplicate. The two methods showed good concurrence ($r^2 = 0.96$).

Lysine analysis

Lysine analysis of the 36 flour samples was performed by the amino acid analysis, the ninhydrin method (section 7.2.4) and by the mOPA method (section 7.2.3). For ninhydrin analysis, samples were assayed 4 times, with the first set discarded due to very low readings. For the mOPA method, 4 protein extractions were performed on each of the flour samples, and each extraction was assayed in triplicate.

Amino acid analysis

Duplicate hydrolysates were prepared for amino acid analyses by weighing 10 mg of ground material in 1.0 mL of redistilled 6 M HCl with 0.1% phenol added. Samples were heated for 24 hours at 110°C in glass tubes sealed under vacuum. Amino acid concentrations were measured using a Waters HPLC ion exchange chromatography system.

7.2.7 Setup of xylose/glucose, pH, dialysis comparison

The study was set up to compare the reaction of RNase A with xylose and glucose, at pH 7 and pH 4 (unadjusted), and with and without dialysis. The incubation was set up such that the RNase A in each sample had come from one original solution, so that carbohydrate type, pH and dialysis were the only variables between samples, as outlined in Figure 7.2-a. Similarly, for all incubations involving the same carbohydrate, the carbohydrate had come from one original solution.

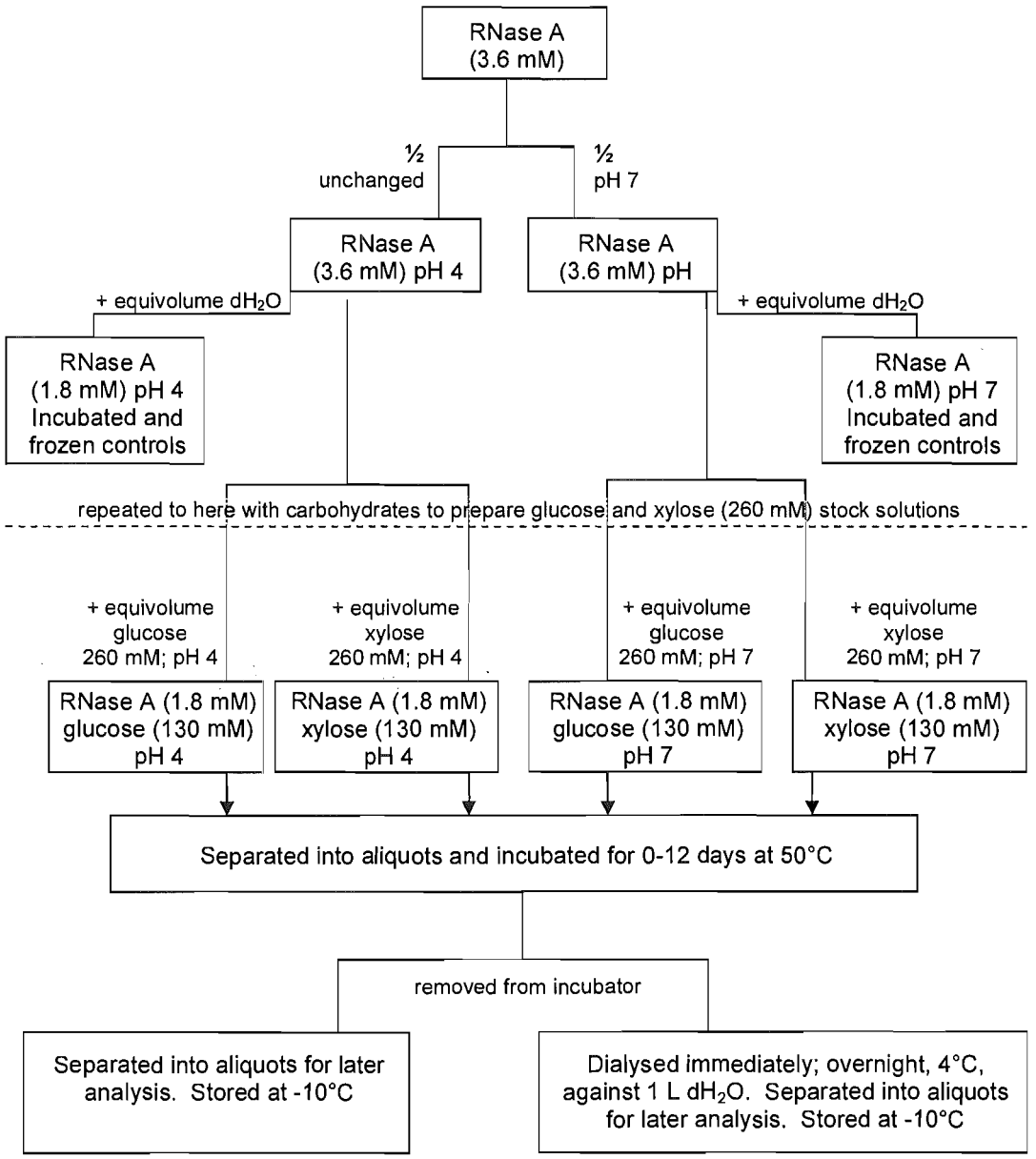

Figure 7.2-a: Overall design of study of pH and dialysis on RNase A incubated with xylose or glucose.

An initial 3.6 mM (50 mg/mL) solution of RNase A was prepared, and the pH of half of the solution adjusted to pH 7. Carbohydrate solutions (274 mM) were prepared in a similar manner. From each of the RNase A solutions (pH 4 and 7) an aliquot was removed, diluted to 1.8 mM, and separated into 4 aliquots, 2 of which were frozen immediately as controls. The 2 remaining RNase A samples, at each pH, were incubated as controls.

Similarly, incubated and frozen carbohydrate controls were prepared for each carbohydrate at each pH (xylose pH 4 and 7; glucose pH 4 and 7). The remaining 3.6 mM RNase A solutions (pH 4 and 7) were separated into equal portions. One pH 4 RNase A portion was combined with an equal volume of 274 mM, pH 4, glucose solution to give a final concentration of protein of 1.8 mM and carbohydrate of 136 mM. Similarly, the remaining three portions of 3.6 mM RNase A solutions were combined with an equal volume of 274 mM carbohydrate solution of the same pH. Each of these carbohydrate/protein solutions were separated into 7 aliquots for subsequent incubation for 0-12 days. After incubation at 50°C, a portion of the sample was dialysed immediately, with the remainder diluted to appropriate concentrations for analyses. Dialysis was carried out overnight at 4°C, against 1 L dH_2O.

Analysis

Protein concentration of dialysed samples was determined using the modified Bradford method (section 7.2.1). SDS-PAGE of all samples was carried out according to the method described in section 7.3.2. Lysine analysis was carried out by the OPA method (section 7.2.2).

7.2.8 Monitoring the Maillard reaction using a pH stat

Following carbohydrate and lysine incubations by pH

Separate solutions of lysine (274 mM) and carbohydrate (274 mM) were prepared, and the pH of each solution was brought up to pH 7 using sodium hydroxide (0.1 mM). The lysine sample was placed in the pH stat, at 37°C and monitored until the pH reading was stable. At this point an equal volume of carbohydrate solution was added, to give a total volume of 1 mL. The change in pH was monitored by the pH stat for 20 hours, with samples maintained at 37°C throughout. Controls were prepared in a similar manner. For lysine and carbohydrate controls, 274 mM solutions were prepared as above, and placed in the pH stat. An equal volume of distilled water was added, and the pH monitored at 37°C. For

distilled water controls, the sample was taken to pH 7, and then the pH was monitored at 37°C.

Following carbohydrate and lysine incubations by addition of sodium hydroxide

Solutions of lysine (264 mM) and carbohydrate (264 mM) were prepared and equal volumes were mixed in the pH stat. The pH was adjusted to pH 7 using sodium hydroxide (0.1 mM). The pH stat then maintained the pH at pH 7 during the course of the reaction by controlled addition of sodium hydroxide. The volume of NaOH required to initially take the pH to 7, and the volume required to maintain the pH at 7 were recorded separately. These incubations were all performed at 37°C, and pH was maintained over 12 hours. Control solutions of carbohydrate or lysine were made up to 137 mM, and then treated as for the lysine/carbohydrate solutions.

7.2.9 Determination of the extent of arginine modification in RNase A by carbohydrate

Arginine availability in RNase A after incubation with carbohydrate was assayed using a microtitre version of a Smith and MacQuarrie method,[8] using 9,10-phenanthrenequinone. RNase A samples were diluted to contain 375 mg of protein per litre of sample. To 100 μL of each diluted sample, 300 μL of 670 μM 9,10-phenanthrenequinone in absolute ethanol and 50 μL of 2 M sodium hydroxide were added. These were then vortexed and incubated at 60°C for 3 hours, with vortexing every half hour. 450 μL of 2 M hydrochloric acid was then added to each sample, which was subsequently vortexed. In duplicate, 300 μL aliquots of each sample were loaded into microtitre plates. Fluorescence was then measured at 395 nm using an excitation wavelength of 312 nm.

7.3 SDS-PAGE

7.3.1 Stock solutions

Stock solutions used in SDS-PAGE were prepared as follows:

Tank buffer

45 g Tris base
216 g glycine
15 g SDS

dH_2O to 3 L

Stored at 4°C.

The solution was diluted 1 in 5 before use.

2 x Treatment buffer

125 µL Tris-HCl (pH 6.8; 1 M)
2.0 mL SDS (10% (w/v))
1.0 mL glycerol (100%)
500 µL 2-mercaptoethanol (100%)
125 µL bromophenol blue (1% (w/v))
750 µL dH_2O

Stored at -10°C.

0.1% Coomassie Brilliant Blue stain

0.1 g Coomassie Brilliant Blue
10 mL glacial acetic acid
50 mL methanol
40 mL dH_2O

The solution was stirred for 20-30 minutes, filtered and stored at room temperature.

Destain

100 mL glacial acetic acid
50 mL methanol (100%)
850 mL dH_2O

Stored at room temperature.

Resolving gel buffer (1.5 M Tris-HCl pH 8.8)

35.3 g Tris base

dH_2O to 200 mL

pH adjusted to 8.8 with HCl.

Stored at 4°C

Stacking gel buffer (0.5 M Tris-HCl pH 6.8)

3.0 g Tris base

dH_2O to 50 mL

pH adjusted to 6.8 with HCl.

Stored at 4°C.

7.3.2 RNase A sample preparation and loading on 8-16% gradient gels

Each sample of RNase A, or RNase A and carbohydrate, was thawed, an aliquot removed and placed in an Eppendorf tube. An equal volume of reducing 2x treatment buffer was added to each sample and the mixture gently vortexed. The samples were placed in a boiling water bath for approximately two minutes to help solubilise the samples and to aid the reduction of disulfide bonds. A 6 μL aliquot of Sigma marker (Table 7.3-a), a wide range molecular weight standard, was also prepared in this way.

Protein	Molecular Weight (kDa)
myosin, rabbit muscle	205
β-galactosidase, E. coli	116
phosphorylase b, rabbit muscle	97
fructose-6-phosphate kinase, rabbit muscle	84
albumin, bovine serum	66
glutamic dehydrogenase, bovine liver	55
ovalbumin, chicken egg	45
glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle	36
carbonic anhydrase, bovine erythrocytes	29
trypsinogen, bovine pancreas	24
trypsin inhibitor, soybean	20
α-lactalbumin, bovine milk	14.2
aprotinin, bovine lung	6.5

Table 7.3-a: The standard molecular weight protein markers constituting Sigmamarker (wide-range).

The wells of a Gradipore pre-cast gel 8-16%, were first rinsed with dH_2O to remove any residual storage buffer. The gel was then set into the electrophoresis rig and the top tank filled with chilled (4°C) 1x tank buffer that had been diluted from a 5x stock (section 7.3.1) until the electrodes were covered. The space surrounding the outside of the gel was filled with 1x tank buffer.

Each boiled protein sample, containing approximately 150 μg of RNase A, and molecular weight marker, were loaded into separate wells in the polyacrylamide gel.

Samples were electrophoresed at a constant current of 150 V at 4°C until the bromophenol blue dye, contained in the 2x treatment buffer, had reached the bottom of the gel. This took approximately 2 hours.

The gel was transferred to a plastic container, then submerged in Coomassie Brilliant Blue stain and placed on an orbital shaker for at least 2 hours, before being transferred into

destain solution. The gel was agitated in destain solution, which was changed at least once, until excess stain had been removed from the gel. The gel was stored in an airtight container, in destain or water, until photographed.

7.3.3 Preparation of 7% and 12% gels

The following method is a modification of the method described in Fayle (1998).[9] The SDS-PAGE of RNase A and cyclotene, shown in section 3.4, was run on a 12% gel. The SDS-PAGE of barley flour samples, shown in section 4.3.4, was run on a 7% gel.

To prepare a gel, two glass plates (22 cm × 22 cm, and 20 cm × 22 cm) were cleaned with Jif and 1-propanol. Two 1.5 mm thick spacers were also cleaned and placed along two sides of the larger glass plate. The smaller plate was then placed on top of the other to form a glass plate sandwich. The glass plates were aligned and clamped, and the SDS-PAGE apparatus assembled according to the manufacturers instructions (Bio-Rad).

Component	**Resolving gel 7%** (7% acrylamide, 0.19% bisacrylamide)	**Resolving gel 12%** (12.2% acrylamide, 0.33% bisacrylamide)	**Stacking gel** (3.5% acrylamide, 0.1% bisacrylamide)
Acrylamide solution	7.2 mL	12.5 mL	1.1 mL
dH_2O	14.8 mL	9.5 mL	5.0 mL
Resolving gel buffer	7.5 mL	7.5 mL	–
Stacking gel buffer	–	-	2.1 mL
10% SDS solution	300 μL	300 μL	820 μL
10% AMPS	150 μL	150 μL	41 μL
TEMED	10 μL	10 μL	20.5 μL
Final Volume	30.0 mL	30.0 mL	9.0 mL

Table 7.3-b: Recipe for the preparation of 7% and 12% SDS-PAGE gels.

The gel solution was prepared according to the recipes in Table 7.3-b. Acrylamide monomer solution, resolving gel buffer, and distilled water were transferred to a clean, dry flask, and mixed under carbon dioxide. SDS, TEMED and freshly prepared AMPS solution

were then added. The flask was swirled to ensure proper mixing. The solution was then slowly poured into the vertical glass sandwich leaving approximately 4 cm at the top for the stacking gel. Polymerisation was allowed to occur overnight, with a layer of water and cling film preventing evaporation and gel shrinkage.

The following day a stacking gel was prepared in the same manner to the resolving gel, and poured on top of the resolving gel. A comb was immediately inserted into the stacking gel mixture, ensuring no air bubbles were trapped beneath it. The assembly was left for approximately 45 minutes prior to loading, while the gel polymerised.

Once polymerised, the vertical glass sandwich containing the gel was slotted onto one side of a central cooling core with the smaller plate facing inwards. Two gels were run per tank, hence another prepared gel was slotted onto the other side of the central cooling core. The upper buffer chamber, formed by the outer large glass plates, was filled with tank buffer. The tank was filled with 1100 mL of tank buffer and 2200 mL of chilled distilled water. The cooling core was placed in the tank, ensuring that no air bubbles were trapped at the base of the gels. The combs were carefully removed from the stacking gels, exposing the sample wells.

7.3.4 RNase A preparation and loading onto 12% gels

RNase A samples were prepared as detailed in section 7.3.2. Each boiled protein sample, containing approximately 250 μg of protein, and 20 μL of treated molecular weight marker, were loaded into separate wells in the polyacrylamide gel.

Samples were electrophoresed at 4°C at a constant current of 48 mA per gel until stacked at the top of the resolving gel. The current was then increased to 72 mA per gel until the bromophenol blue dye, contained in the 2x treatment buffer, had reached the bottom of the gel. This took approximately 5 hours.

Gel was stained with Coomassie Brilliant Blue and destained as detailed in section 7.3.2.

7.3.5 Total flour protein extract preparation and loading onto 7% gels

Whole flour protein extracts were thawed and, if cloudy, heated for five minutes at 60°C. An aliquot was removed from each of the samples and placed in an Eppendorf tube. An equal volume of reducing 2x treatment buffer was added to each sample and the mixture gently vortexed. The samples were placed in a boiling water bath for approximately two minutes to solubilise the samples and to aid the reduction of disulfide bonds. Sigmamarker (Table 7.3-a), a wide-range molecular weight standard, was also treated in this way.

80 μL of each boiled protein sample and 12 μL of treated molecular weight marker were loaded into separate wells in the polyacrylamide gel.

Samples were electrophoresed at 4°C at a constant current of 16 mA per gel until stacked at the top of the resolving gel. The current was then increased to 25 mA per gel until the bromophenol blue dye, contained in the 2x treatment buffer, had reached the bottom of the gel. This took approximately 5-8 hours.

Gel was stained with Coomassie Brilliant Blue and destained as detailed in section 7.3.2.

7.3.6 Total protein extract preparation and loading onto 4-20% gels

Whole flour protein extracts were treated as described in section 7.3.5. Each boiled protein sample, containing approximately 7 μL of protein extract, and 12 μL of treated molecular weight marker, were loaded into separate wells in the polyacrylamide gel.

Samples were electrophoresed at 4°C at 150 V until the bromophenol blue dye, contained in the 2x treatment buffer, had reached the bottom of the gel. This took approximately 1½ hours.

Gel was stained with Coomassie Brilliant Blue and destained as detailed in section 7.3.2.

7.3.7 Chicken feed extract preparation and loading onto 4-20% gels

Chicken feed protein extracts were treated as described in section 7.3.5. Each boiled protein sample, containing approximately 4 μL of protein extract, and 12 μL of treated molecular weight marker, were loaded into separate wells in the polyacrylamide gel.

Samples were electrophoresed as detailed in section 7.3.6. The gel was stained with Coomassie Brilliant Blue and destained as detailed in section 7.3.2.

7.4 EXPERIMENTAL FOR WORK DESCRIBED IN CHAPTER 3 – INVESTIGATING THE MAILLARD REACTION IN MODEL SYSTEMS

7.4.1 RNase A incubations with cyclotene

The following is based on a method described by Fayle *et al.*[10] RNase A (25 mg/mL; 1.8 mM) was mixed with cyclotene (25 mg/mL; 2.2 M). An RNase A frozen control, containing no cyclotene, was placed immediately on ice and stored at -10°C. The samples and two incubated controls containing only RNase A were incubated at 37°C, and taken out after 5, 12, 15, 20, 25, 30 and 35 days. Samples were dialysed against 1 L distilled water overnight, and then stored at -10°C. This incubation was run in duplicate.

A further replicate was run, with the pH was adjusted to 7.0, as determined by pH meter, prior to incubation. Incubations were removed at between 0 and 35 days.

Analysis

RNase A incubations were analysed by SDS-PAGE (section 7.3.2) and the OPA method (section 7.2.2) for determining lysine availability. Arginine availability was determined using a microtitre version of Smith and MacQuarrie's 9,10-phenanthrenequinone method (section 7.2.9).[8] Protein concentrations were estimated using a modified Bradford method (section 7.2.1).[2,3]

7.4.2 RNase A incubations with various carbohydrates

A set of incubations was carried out with RNase A and eight carbohydrates. The carbohydrates cyclotene, glucose, xylose and sucrose were incubated at concentrations of 130 mM. The carbohydrates dextrin, soluble starch, malt extract and molasses were incubated at concentrations of 25 mg/mL. RNase A (1.8 mM, 25 mg/mL) was incubated with each carbohydrate separately. The starting pH for all solutions was 7, as measured by pH meter. A zero-time sample for each RNase A/carbohydrate sample, and 3 RNase A only frozen control were placed immediately on ice and stored at -10°C. Three sets of incubations, at 37°C, 50°C and 70°C, were undertaken for each carbohydrate. Incubations were removed after 12 hours and after 1, 2, 4, 6 and 8 days for incubations at 37°C and 50°C. For incubations at 70°C, samples were taken out after 12 hours, and after 1, 3 and 5 days. Two RNase A only controls were also incubated, at each experimental temperature, for the duration of the experiment. Samples were stored at -10°C.

Incubations were run in duplicate.

Analysis

RNase A incubations were analysed by SDS-PAGE (section 7.3.2) and the OPA method (section 7.2.2) for determining lysine availability.

7.5 EXPERIMENTAL FOR WORK DESCRIBED IN CHAPTER 4 – LYSINE LOSS DURING THE PELLETING PROCEDURE

7.5.1 Effect of carbohydrate type on crosslinking and lysine content

Incubations containing barley flours in the presence of various carbohydrates were prepared according to the mixtures shown in Table 7.5-a.

Sample	Carbohydrate	Autoclaved	Flour	Carbohydrate
Fc	None	no	100%	-
F	None	yes	100%	-
FX	Xylose	yes	67%	33%
FB	Molasses	yes	67%	33%
FS	Starch	yes	67%	33%
FM	Malt extract	yes	67%	33%
FD	Dextrin	yes	67%	33%

Table 7.5-a: Composition of autoclaved and control samples for modelling of the effect of pelleting on flour, carbohydrate and lysine mixes.

All flour samples contained 1 g of barley flour and, if present, 500 mg of carbohydrate. For each incubation, the appropriate ingredients were weighed out, placed into a small beaker, mixed thoroughly then placed into McCartney bottles. The lids were screwed loosely onto the bottles, prior to autoclaving. Standard wet run autoclave conditions were used. After the autoclave run, the samples were stored at -10°C until analysis. Controls not autoclaved were placed immediately into storage at -10°C.

Analysis

Total protein was extracted from each sample, as outlined in section 7.2.5, and then analysed by SDS-PAGE as described in section 7.3.6. Lysine analysis was carried out, using mOPA method (section 7.2.3). Interference from brown Maillard reaction products was accounted for using 50% 1-propanol solutions containing an equivalent amount of sample.

Absorbance of 6% total flour protein extracts at 460 nm were recorded.

7.5.2 Effect of lysine addition on crosslinking and lysine content

Incubations containing barley flours in the presence of xylose and/or lysine were prepared according to the mixtures shown in Table 7.5-a. For each incubation, the appropriate ingredients were weighed out, placed into a small beaker, mixed thoroughly then split into 2 equal portions. One portion was placed into a glass vial for freezing as a control. The remainder was placed into a McCartney bottle for autoclaving. The lids were screwed loosely onto the bottles, prior to autoclaving. After the autoclave run, the samples were stored at -10°C until analysis. Controls not autoclaved were placed immediately into storage at -10°C.

Sample	Flour	Carbohydrate	Lysine
L	-	-	100%
X	-	100%	-
F	100%	-	-
FL	99%	-	1%
FX	95%	5%	-
FXL	94%	5%	1%

Table 7.5-b: Composition of autoclaved and control samples for modelling of the effect of pelleting on flour, carbohydrate and lysine mixes.

Analysis

Total protein was extracted from each sample, as outlined in section 7.2.5, and then analysed by SDS-PAGE as described in section 7.3.6. Lysine analysis was carried out, using mOPA method (section 7.2.3). Interference from brown Maillard reaction products was accounted for using 50% 1-propanol solutions containing an equivalent amount of sample.

Absorbance of 6% total flour protein extracts at 460 nm were recorded.

7.5.3 Lysine loss during pelleting – standard feed

Initial sample collection and treatment

Broiler finisher 1 (F1) finisher feed mixes were provided by Tegel Foods Ltd. On the first collection date, three samples were collected from a standard pelleting run. These three samples were comprised of standard meal (prior to entering the pelleting process), ex-conditioner (after the first heating stage of the pelleting process) and pellets as exiting the pelleting process. The ex-conditioner sample was immediately placed in liquid nitrogen for transportation. All samples were then stored at -10°C.

Milling

Samples were milled to a maximum diameter of either 1 mm or 0.5 mm. The mill was thoroughly cleaned between samples. The initial 5 g of each milling run was discarded to minimise the possibility of cross-contamination of samples.

Extraction of protein and free amino acids

Standard extraction procedure from section 7.2.5 adapted as follows:

Single extraction into water. Standard procedure followed, but dH_2O used in place of standard extraction solution.

Double extraction – First extraction – standard procedure followed. Second extraction – sample pellet resuspended by vortex mixing in second 400 µL aliquot of extraction solution. Standard procedure followed for extraction. Two 400 µL extract aliquots combined for subsequent analysis.

Single extraction into 500-800 µL. Standard procedure followed, but 500-800 µL of extraction solution used.

Water extraction at 60°C – standard procedure followed, but extraction solution replaced by 800 – 1000 µL of distilled water.

Water extraction at room temperature – standard procedure followed, but extraction solution replaced by 800 – 1000 µL of distilled water, and carried out without heating.

Analysis

Replicate extractions (3-10) were performed on sets of meal, ex-conditioner and pellet samples *via* the standard method and the methods outlined above. Due to some samples becoming cloudy on freezing, all lysine analysis was performed on the same day as extraction. Lysine analysis was carried out by the mOPA method (section 7.2.3) for extracts into standard solution, and by the OPA method (section 7.2.2) for extracts into water, in triplicate for each extraction. Lysine was used as the standard for these methods.

SDS-PAGE was performed as detailed in section 7.3.7 using standard and (a) sample extractions. Equivolumes of each extraction was loaded onto the gel.

7.5.4 Lysine loss during pelleting – feeds with various amino acids not added to the mix

At the second collection date, samples prepared in trial feed runs by the Tegel feed mill were collected. These trial runs consisted of 1 tonne pellet processing with various amino acids not added to the meal prior to pelleting (Table 7.5-c). Meal, ex-conditioner and pellets were collected and stored at -10°C.

Sample Label	Lysine	Methionine	Threonine
LMT	✓	✓	✓
MT	X	✓	✓
LT	✓	X	✓
LM	✓	✓	X
L	✓	X	X

Table 7.5-c: Amino acids added to sample feeds made up at Tegel Feed Mill.

7.5.5 Sample treatment and analysis

10 samples of 50 and 60 mg of each meal, ex-conditioner and pellet trial feed was weighed accurately into an Eppendorf tube. To each tube, 1 mL of distilled water was added, and then incubated at room temperature for 30 minutes, with vortex mixing every 5 minutes. The samples were then centrifuged at 4300 *g* for 4 minutes. The supernatant was decanted and diluted by a factor of 3 to bring lysine content into an appropriate range to be assayed. Lysine analysis was carried out on the same day as this treatment was performed. Protein in the sample extracts were analysed for lysine content using the OPA method as outlined in section 7.2.2. Lysine was used as the standard for calibration of this method. SDS-PAGE was performed as detailed in section 7.3.7.

7.5.6 Efficacy of TGase in model flour systems

Incubations looking at the effect of autoclaving on barley flours in the presence of various carbohydrates and lysine were set up according to the mixtures shown in Table 7.5-d.

Sample	Autoclaved	Flour	Xylose	TGA	dH_2O
Fc	No	44%	22%	-	33%
Fd	Yes	100%	-	-	-
Fw	Yes	67%	-	-	33%
FTd	Yes	100%	-	0.3%	-
FTw	Yes	67%	-	0.2%	33%
FXd	Yes	67%	33%	-	-
FXw	Yes	44%	22%	-	33%
FXTd	Yes	67%	33%	0.2%	-
FXTw	Yes	44%	22%	0.1%	33%

Table 7.5-d: Composition of autoclaved and control samples for modelling of the effect of TGase in pelleting on flour, xylose and lysine mixes.

All flour samples contained 1 g of barley flour, and the appropriate mass of other ingredients. All ingredients were weighed out, placed into small beakers with the other ingredients for that sample, mixed thoroughly and placed into a McCartney bottle. The lids

of the McCartney bottles were then screwed on loosely, and samples left to sit for approximately 2 hours prior to autoclaving. After the autoclave run, the samples were stored at -10°C until analysis. Controls not autoclaved were placed immediately into storage at -10°C.

Analysis

All samples were freeze dried. Total protein was extracted from each freeze dried sample, as outlined in section 7.2.5, and then analysed by SDS-PAGE as described in section 7.3.3. For this gel, 40 µL of protein extract was loaded. Browning of total protein extracts was assessed by measuring the absorbance of at 460 nm.

7.6 EXPERIMENTAL FOR WORK DESCRIBED IN CHAPTER 5 – LYSINE LOSS DURING FEED PROCESSING: BROILER GROWTH TRIALS

7.6.1 Trial procedure

The feeding trial took place at the Monogastric Unit at Massey University. 600 (male) just hatched birds were supplied by the Tegel Levin hatchery on the 9th January 2001, and fed standard Tegel starter diet until 7 days old. At this point, the birds were weighed, and outliers were removed from the trial. The remaining 448 birds were placed into groups of seven birds, to give groups with the same approximate group weights. These groups were placed into the trial cage facility, and randomly assigned to the trial diets, to give eight replicate groups of seven birds on each of the eight trial diets. Trial diets were fed to the birds from when they were 7 to 21 days old. Trial diet composition and preparation are outlined in section 7.6.2.

Throughout the trial, feed and water were available *ad libitum*, and standard temperature regimes were followed (35°C-29°C).

At the same times each required day, and with the same sequences and routines, the following measurements were made at weekly intervals (*i.e.* when the birds were 7, 14 and 21 days old):

- Live group weight. At each weighing, the first two pens were reweighed as a control.
- Feed consumption per group.
- Mortality (recorded daily).

7.6.2 Feed preparation

Standard F1 feeds were prepared in the normal manner at the New Plymouth and the Christchurch Tegel feed mills. The New Plymouth feed mill also prepared an F1 feed that contained no synthetic lysine. These were transported to the Poultry Research Unit at Massey University for the feed trial. The feed that did not contain synthetic lysine was split into six parts of 80 kg each. Each part was placed in a rotary mixer, and was sprayed with one litre of lysine solution, or water, to give 0 - 4.37 kg/tonne of the hydrochloric salt of lysine, as shown in Table 7.6-a. These mixes were then stored for one to three weeks prior to feeding to the chicks, which arrived as day-olds on the day of the feed mixing.

Feed Label	**Feed Source***	**Lysine-HCl** added pre-pelleting (kg/tonne)	**Lysine-HCl** added post-pelleting (kg/tonne)
T1	NP	4.400	–
T2	ChCh	3.900	–
T3	NP	–	–
T4	NP	–	0.875
T5	NP	–	1.750
T6	NP	–	2.620
T7	NP	–	3.500
T8	NP	–	4.370

* NP – New Plymouth Feed Mill; ChCh – Christchurch Feed Mill

Table 7.6-a: Lysine-HCl content of trial feeds.

7.6.3 Feed sample collection

First Sample Collection

The first sets of pellets were collected from the Palmerston North Poultry Research Unit. These samples consisted of all New Plymouth sourced feeds – T1, T3 to T8. Four 100 g portions of each sample were collected from the rotary mixer after liquid addition, for samples T3-8, or from the feed bags for sample T1. These samples were not frozen until 3 days post collection as the bag containing the samples was misplaced by the airline during the Palmerston North to Christchurch flight.

Four 200 g portions of T2 sample were collected on arrival at the Massey facility, and picked up on the second sample collection date. These were frozen on arrival in Christchurch.

Second Sample Collection

The second sample collection date was the final day of the feed trial. Samples were collected from undistributed feed, which was still in the storage bags. This feed had been stored in the warehouse for two weeks and in the trial facility for one week, and was equivalent to what the birds were fed on the final day of the trial. These samples, originally labelled T1 to T8, were labelled E1-E8 respectively, and frozen on arrival in Christchurch the same day.

Samples were also collected from the feed remaining in the troughs on the final day of the trial. These feeds were transferred into bags for each cage, and weighed as per normal trial procedure. Small samples (~ 70 g) were then taken from each weighed bag of feed, and pooled for each feed type. These were also frozen on arrival in Christchurch the same day. These samples were later sieved through a 1 mm diameter sieve to separate pellets and coarse material from pellet fines. The coarse material from samples T1-8 were labelled cL1-cL8 respectively. Pellet fines were labelled fL1-fL8 respectively.

7.6.4 Sample analysis

All samples were ground to 0.5 mm maximum diameter prior to analysis. Extraction and lysine analysis was performed as described in section 7.5.5. SDS-PAGE was performed on protein extracts as detailed in section 7.3.7. Moisture contents for each sample were determined by measuring 3 g of each sample into weighed McCartney bottles. These bottles were then placed in a drying oven at 60°C for 24 hours, and reweighed.

7.6.5 Statistical analysis

Statistical analysis of samples was performed using Minitab (version 14). Correlations were performed using one-way ANOVA analysis, with outliers, as identified by Minitab, removed from analysis.

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Appendix I

Calculating lysine concentration, as eaten, per bird, per diet

Abbreviations

L = Lysine [d]

tF = Total feed given per bird [e]

eF = Feed eaten per bird [b]

uF = Feed uneaten per bird [b]

fuF = Fine feed uneaten per bird = uF x 23% [b,c]

cuF = Coarse feed uneaten per bird = uF x 77% [b,f]

For each diet the following calculations were made:

L in diet = L eaten per bird / eF
g/kg g kg

L eaten per bird = total L fed per bird – L uneaten per bird
g g g

total L fed per bird= tF x g L/kg in "As Fed"
g kg g/kg

L uneaten per bird = L in "Coarse" per bird + L in "Fines" per bird
g g g

L in "Coarse" per bird = cuF x g L/kg in "Coarse"
g kg g/kg

L in "Fines" per bird = fuF x g L/kg in "Fines"
g kg g/kg

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[d] Values for lysine concentrations (g L/kg) for "Coarse", "Fines" and "As Fed" taken from Table 5.5a.

[e] Data was collected per cage of birds. Per bird data was calculated using the average number of birds in the cage over the period of the trial, taking into account mortality information.

[f] Value of 23% found as described in section 5.4.3; value of 77% from difference *i.e.* 100% – 23%

Appendix II

Data for the Growth Trial described in Chapter 5

Growth (g) (standard error)						
Diet	**Week A**		**Week B**		**Overall**	
1	325	(5)	461	(6)	786	(10)
2	308	(5)	456	(2)	764	(7)
3	271	(4)	446	(6)	717	(6)
4	283	(5)	452	(4)	735	(6)
5	288	(5)	471	(10)	751	(9)
6	301	(5)	454	(6)	756	(8)
7	303	(5)	459	(5)	761	(5)
8	314	(4)	462	(5)	776	(8)

Feed (g/bird) (standard error)						
Diet	**Week A**		**Week B**		**Overall**	
1	430	(5)	688	(8)	1121	(11)
2	440	(4)	700	(7)	1140	(11)
3	408	(6)	703	(8)	1110	(10)
4	405	(6)	680	(7)	1086	(6)
5	413	(3)	694	(7)	1107	(6)
6	417	(5)	678	(7)	1095	(10)
7	424	(6)	691	(6)	1115	(8)
8	425	(5)	684	(5)	1106	(7)

FCR (standard error)						
Diet	**Week A**		**Week B**		**Overall**	
1	1.31 [a]	(0.01)	1.47 [a]	(0.02)	1.41 [a]	(0.02)
2	1.43 [b]	(0.02)	1.53 [b]	(0.01)	1.49 [b]	(0.01)
3	1.49 [b]	(0.01)	1.57 [c]	(0.02)	1.53 [c]	(0.01)
4	1.44 [b]	(0.02)	1.51 [c]	(0.01)	1.48 [d]	(0.01)
5	1.44 [b]	(0.02)	1.48 [c]	(0.03)	1.46 [d]	(0.02)
6	1.39 [c]	(0.01)	1.51 [c]	(0.01)	1.46 [d]	(0.01)
7	1.40 [c]	(0.01)	1.50 [c]	(0.02)	1.46 [d]	(0.01)
8	1.36 [c]	(0.01)	1.50 [c]	(0.01)	1.46 [d]	(0.01)

Superscripts denote samples with differences significant at $P<0.05$

Mortality			
Diet	**Week A**	**Week B**	**Overall**
1	0.0%	1.8%	1.8%
2	0.0%	0.0%	0.0%
3	0.0%	0.0%	0.0%
4	0.0%	1.8%	1.8%
5	0.0%	1.8%	1.8%
6	0.0%	0.0%	0.0%
7	1.8%	1.8%	3.6%
8	0.0%	1.8%	1.8%

Appendix III

Follow-up Growth Trial

As a consequence of the finding that lysine becomes less necessary in the third week of the bird's life (chapter 5), Tegel Food Ltd. performed a follow-up growth trial to determine the effect of diet form on lysine requirements over this period. In this trial, a wheat/barley/soy based diet was formulated and half was supplemented with lysine, to a degree calculated to be optimal for growth, and then pelleted. The remaining feed was not supplemented with free lysine and was not pelleted. To each of these base diets, 4 different levels of lysine were added post-pelleting (Table A). In terms of this thesis, involvement was limited to testing samples, collected at 1 week intervals for a total of 3 weeks, for lysine content. The lysine contents were used to revalidate the methodology used to measure lysine contents of chicken feed.

Diet	Lysine-HCl added			Total lysine added[a] (g/kg)
	Pre-pelleting	***Post-pelleting***	***Total*** (g/kg)	
m	0.34%	0%	3.20	2.56
n	0%	0%	0	0
o	0.34%	0.085%	4.05	3.24
p	0.34%	0.170%	4.90	3.92
q	0.34%	0.340%	6.60	5.28
r	0%	0.085%	0.85	0.68
s	0%	0.170%	1.70	1.36
t	0%	0.340%	3.40	2.72

[a] Calculated from total lysine-HCl addition by method detailed in section 5.4.2

Table A: Theoretical amounts of lysine added pre and post-pelleting to trial diets in the follow-up growth trial.

Samples were tested for water soluble lysine in the manner described for the above growth trial. Results showed little variation over the sampling period, and hence data from the three weeks was collated for correlation with the theoretical added lysine values, as shown in Figure A.

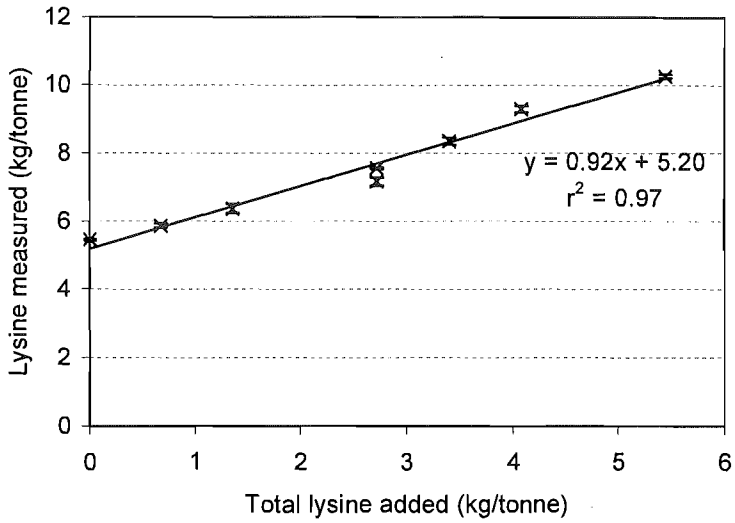

Figure A: Comparison between measured and added values for lysine. Data points are average values of 15 extractions (5 extractions for each diet, sampled three times over three weeks). Error bars represent standard error of these 15 replicates.

As seen in the previous study, the theoretically added lysine correlated extremely well with the measured lysine values, showing the methodology was valid. A difference of 8%, between theoretical and measured lysine content values was seen, but this was much lower than the 19% difference observed in the previous trial (Figure 5.4-b). As it is unlikely that the feed samples from this trial were subjected to significantly different conditions to the previous trial, and hence should have undergone a similar degree of Maillard reaction, this suggests that the lysine used in the previous trial may have been weighed incorrectly in the prior trial (section 5.4.2).